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LEUKOCYTE RECRUITMENT AND CONTROL OF VASCULAR PERMEABILITY IN ACUTE INFLAMMATION

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"INFLAMMATION IN ITSELF IS NOT TO BE CONSIDERED AS A DISEASE,
BUT AS A SALUTARY OPERATION
CONSEQUENT EITHER TO SOME VIOLENCE OR DISEASE"

– John Hunter, A Treatise of the Blood, Inflammation, and Gunshot Wounds, 1794.

To My Family

ABSTRACT

The inflammatory process is fundamental in host defense against tissue injury or infection. However, the inflammatory reaction may itself cause harm to the host and contribute to tissue damage and organ dysfunction. Leukocyte recruitment and edema formation are key components of the inflammatory response. This thesis reports experiments that were undertaken to further elucidate the mechanisms controlling leukocyte extravasation and concurrent alteration of vascular permeability in acute inflammation.

In order for leukocytes to penetrate the vessel wall they need to sequentially interact with the endothelial lining and the perivascular basement membrane (BM) of which laminin-411 is a major constituent. The role of BM laminin-411 in leukocyte recruitment to inflammatory loci was addressed using α4 chain deficient (Lam4-/-) and wild-type (WT) mice. Recruitment of all major leukocyte subsets (neutrophils, monocytes, and lymphocytes) was reduced in Lam4-/- mice compared to WT. With the use of intravital microscopy it was concluded that this decrease was due to impaired diapedesis through the vessel wall.

Concurrent with neutrophil recruitment to extravascular tissue, there is an increase in vascular permeability. However, the mechanism behind this alteration is unknown. It was shown that stimulation of neutrophils with the potent chemoattractant leukotriene B_4 (LTB_4) leads to degranulation and release of, amongst others, heparin binding protein (HBP). Further, postsecretory supernatants from LTB_4-stimulated neutrophils induced intracellular calcium mobilization in endothelial cells *in vitro* and increase in vascular permeability *in vivo*. Selective removal of HBP from the supernatant significantly reduced these activities indicating a role for HBP in LTB_4-induced plasma extravasation. The mechanism behind neutrophil-induced alteration of endothelial barrier function was further investigated and revealed a pivotal role of the kallikrein-kinin system. Neutrophil activation was shown to enable proteolytic processing of high molecular weight kininogen bound to endothelial cells. Accordingly, plasma exudation *in vivo* in response to challenge with leukocyte chemoattractants was largely annulled by antagonists of the kallikrein-kinin system. Collectively, the data provide novel insight into the regulation of neutrophil-induced plasma extravasation and may help to identify better therapeutic strategies for interventions in inflammatory disease.

To investigate the role of neutrophil-induced alterations in vascular permeability in a clinically relevant setting, experiments were performed using controlled cortical impact (CCI) as a model for traumatic brain injury (TBI) in normal mice and in mice that were depleted of neutrophils. Neutrophil depletion did not significantly affect plasma leakage across the blood-brain barrier after CCI. Yet, neutrophils were found to play a role in edema formation in brain tissue after injury. At a later phase, neutropenic mice displayed a decreased number of activated microglia, and an attenuation of tissue loss after injury. These results suggest that neutrophils contribute to the secondary injury following TBI. Altogether, this thesis provides insight into the role of the BM in leukocyte recruitment and clarifies the mechanism behind neutrophil-induced edema formation in acute inflammation.

LIST OF PUBLICATIONS

The thesis is based on the following articles, which are referred to in the text by their Roman numerals:

I **Kenne E**, Soehnlein O, Genové G, Rotzius P, Eriksson EE, Lindbom L. (2010)
Immune cell recruitment to inflammatory loci is impaired in mice deficient in basement membrane protein laminin α4
J Leukoc Biol. In press

II Di Gennaro A, **Kenne E**, Wan M, Soehnlein O, Lindbom L, Haeggström JZ. (2009)
Leukotriene B4-induced changes in vascular permeability are mediated by neutrophil release of heparin-binding protein (HBP/CAP37/azurocidin)
FASEB J. 23:1750-1757.

III **Kenne E**, Renné T, Soehnlein O, Muller-Esterl W, Flodgaard H, Herwald H, Lindbom L.
Neutrophil-induced alterations in vascular permeability – Role of the kallikrein-kinin system
Manuscript

IV **Kenne E**, Erlandsson A, Hillered L, Lindbom L, Clausen F.
Neutrophils contribute to the edema formation following head trauma
Manuscript

Publications by the author, which are not included in the thesis:

Rotzius P, Thams S, Soehnlein O, **Kenne E**, Tseng CN, Björkström NK, Malmberg KJ, Lindbom L, Eriksson EE (2010). Distinct infiltration of neutrophils in lesion shoulders in ApoE-/- mice. *Am J Pathol.* 177:493-500.

Rotzius P, Soehnlein O, **Kenne E**, Lindbom L, Nystrom K, Thams S, Eriksson EE (2.ApoE(-/-)/lysozyme M(EGFP/EGFP) mice as a versatile model to study monocyte and neutrophil trafficking in atherosclerosis (2009). *Atherosclerosis*. 202:111-8.

Soehnlein O, Kai-Larsen Y, Frithiof R, Sorensen OE, **Kenne E**, Scharffetter-Kochanek K, Eriksson EE, Herwald H, Agerberth B, Lindbom L (2008). Neutrophil primary granule proteins HBP and HNP1-3 boost bacterial phagocytosis by human and murine macrophages. *J Clin Invest*. 118:3491-502.

Gorfu G, Virtanen I, Hukkanen M, Lehto VP, Rousselle P, **Kenne E**, Lindbom L, Kramer R, Tryggvason K, Patarroyo M (2008). Laminin isoforms of lymph nodes and predominant role of alpha5-laminin(s) in adhesion and migration of blood lymphocytes. *J Leukoc Biol*. 84:701-12.

Soehnlein O, **Kenne E**, Rotzius P, Eriksson EE, Lindbom L (2008). Neutrophil secretion products regulate anti-bacterial activity in monocytes and macrophages. *Clin Exp Immunol*. 151:139-45.

Soehnlein O, Xie X, Ulbrich H, **Kenne E**, Rotzius P, Flodgaard H, Eriksson EE, Lindbom L (2005). Neutrophil-derived heparin-binding protein (HBP/CAP37) deposited on endothelium enhances monocyte arrest under flow conditions. *J Immunol*. 174:6399-405.

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INTRODUCTION

INFLAMMATION

Inflammation is the body's protective response to foreign and noxious stimuli and which serves to eliminate the initial cause of cell injury as well as the necrotic cells resulting from the original insult. The inflammatory response also has a role in healing and reconstitution of tissue. It involves the coordinated response of blood vessels, leukocytes and plasma components. Vascular and cellular changes are triggered by soluble factors that are produced by various cells or derived from plasma proteins as a response to the inflammatory stimulus. Inflammation usually terminates when the initiating stimulus disappear. However, sometimes inflammation may be inappropriately triggered or not adequately controlled and may then cause harm to the host. Examples of these conditions include chronic inflammatory diseases such as rheumatoid arthritis, atherosclerosis and fibrosis of the lung as well as acute conditions such as sepsis (Kumar & Robbins, 2009).

The inflammatory response involves several components:

- Circulating cells such as polymorphonuclear leukocytes (neutrophils, eosinophils and basophils), lymphocytes, monocytes and platelets, and plasma proteins including clotting factors, kininogens and complement components.
- The vascular wall consisting of the endothelium, basement membrane, pericytes and smooth muscle cells.
- The extracellular matrix hosting mast cells, macrophages and dendritic cells (Kumar *et al.*, 2003).

Microcirculatory Changes in Inflammation

The four cardinal signs of inflammation were listed by Celcius around 100 AD and are *rubor* (redness), *tumor* (swelling), *calor* (heat) and *dolor* (pain). Rudolf Virchow added the fifth sign; loss of function (*functio laesa*) in the 19th century. These signs occur as a result of adaptive changes in the microcirculation, namely heat and redness due to arteriolar dilatation and increased local blood flow, and swelling of the tissue due to increased vascular permeability and consequent plasma leakage from postcapillary venules.

Acute and chronic inflammation

The inflammatory response can be divided into an acute and a chronic pattern. However, these forms of inflammation frequently overlap. Acute inflammation is of short duration (minutes to days) and is characterized by accumulation mainly of neutrophilic granulocytes (see later section) and concurrent exudation of fluid and plasma proteins. Blood flow to the affected area is increased by upstream vasodilation. The endothelium is affected to decrease its barrier function, which leads to an efflux of plasma resulting in edema formation. Neutrophils emigrate from the microvasculature to the area of injury or infection (Kumar *et al.*, 2003). Acute

inflammation can be triggered by several factors; infections and microbial toxins, physical and chemical injury, tissue damage resulting from ischemia or trauma, and immunological reactions.

Chronic inflammation is of longer duration (days to years) and involves primarily mononuclear leukocytes (monocytes and lymphocytes) and macrophages with associated vascular proliferation and scarring. A chronic inflammatory condition is prolonged and has simultaneously occurring active inflammation, tissue destruction largely directed by inflammatory cells, and repair which involves angiogenesis and fibrosis. Acute inflammation can progress to chronic if the injurious agent is persistent or if the normal healing process is disturbed. Examples of chronic conditions include persistent microbial infections or autoimmune disorders such as rheumatoid arthritis or multiple sclerosis (Kumar *et al.*, 2003). This thesis focuses on the vascular changes and cellular events during acute inflammation.

Cells of the immune system

Immune cells are classified either as bloodborne or tissue residing. Based on histological appearance, the bloodborne cells are further distinguished as either polymorphonuclear or mononuclear. Polymorphonuclear leukocytes (PMN) include *neutrophilic granulocytes* (neutrophils) that are recruited during acute inflammation to phagocytose microbes; *eosinophilic granulocytes* that are recruited during allergic inflammatory reactions and parasitic infections; and *basophilic granulocytes* that participate in allergic inflammation. Monocytes and lymphocytes make up the mononuclear cells. *Monocytes* are recruited from the blood during later stages of acute inflammation and during chronic inflammation, and differentiate into macrophages in the extravascular tissue where they act as phagocytes. *Lymphocytes* are specialized cells of the adaptive immune system that act as directors of antigen-specific immune responses including the synthesis of antibodies. Tissue residing immune cells include *mast cells* that produce lipid mediators and histamine, and *macrophages* that produce cytokines and function as effector cells in cell-mediated immunity (Abbas & Lichtman, 2009).

The neutrophil

Neutrophils constitute the predominant cell type early in the inflammatory reaction as they are more numerous in the blood than monocytes and react more rapidly to chemokines (Kumar & Robbins, 2009). They respond especially to bacterial and fungal infections, and die after a few hours in the tissue. With the help of reactive oxygen species (ROS) they phagocytose and destroy microbes. Neutrophils recognize antigen in the blood and extravascular tissue using several types of receptors such as toll like receptors, and receptors for formyl methionine peptides and products of complement activation (Abbas & Lichtman, 2009). The cytoplasm is filled with four types of granules that are formed in a specific sequence during differentiation in the bone marrow (Borregaard *et al.*, 2007):

- Primary or azurophilic granules are formed during the early stage of PMN differentiation and contain bactericidal and cytotoxic mediators such as myeloperoxidase (MPO), which catalyzes the formation of hypochloric acid

from hydrogen peroxide and is very toxic for microbes; defensins and bacterial permeability increasing protein, and the serine proteases elastase, cathepsin G, proteinase 3, and heparin binding protein (HBP/ azurocidin). The serine proteases are cationic glycoproteins of similar size.

- Secondary or specific granules, which are created after the primary granules are the most common. They contain bactericidal proteins such as lactoferrin, LL-37 and lysozyme, as well as NAPDH-oxidase, which is involved in the production of ROS.
- Tertiary or gelatinase granules contain matrix metalloproteases (MMPs) that degrade extracellular matrix proteins.
- Secretory vesicles contain complement receptors and integrins (i.e. CD11/CD18) as well as plasma proteins. They act as storage of membrane proteins that can be mobilized rapidly during activation (Borregaard *et al.*, 2007). The inactive serine protease HBP is also found in the secretory vesicles (Tapper *et al.*, 2002).

The granules are mobilized as a response to neutrophil activation in a hierarchical order starting with the secretory vesicles, tertiary granules and secondary granules. The secretory vesicles are released during the contact between the PMN and endothelial cell (EC) as a result of selectin signaling or by inflammatory mediators on the endothelium (Borregaard *et al.*, 2007). The release results in increased adhesion via β_2-integrins. The tertiary granules contain MMPs which are able to degrade collagen IV in the basement membrane (BM) and it is likely that migration through the BM requires the release of these granules (Reichel *et al.*, 2008). Primary and secondary granules are released when the neutrophil has emigrated to the extravascular tissue and contribute to the reactive oxygen-dependent and -independent bactericidal activity. The degree to which the granules are released is determined by the strength of the inflammatory stimulus. A strong stimulus will lead to high enough intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) to induce the release of primary granules (Lacy & Eitzen, 2008).

The degranulation process can occur extracellularly or intracellularly and is initiated upon receptor-mediated stimulation of PMN. The release of granules is dependent on Ca^{2+} and to a certain extent hydrolysis of ATP (Theander *et al.*, 2002). ATP acts as an energy source for SNARE complex reorganization and Ca^{2+} is required for cytoskeletal activation. Actin is found to associate with all granule subsets, which indicates its importance in the control of degranulation (Jog *et al.*, 2007). Neutrophils express three Src family members that, when activated by receptor ligation, control granule release (Mocsai *et al.*, 2000). Finally, the Rho subfamily of GTPases, which regulates actin cytoskeletal rearrangement, is also involved in neutrophil exocytosis (Lacy & Eitzen, 2008).

Inflammatory mediators

The major cell types that produce substances mediating the acute inflammatory reaction are platelets, neutrophils, monocytes/macrophages and mast cells. Inflammatory mediators are also derived from plasma proteins, e.g. kinins and complement factors, and are produced mainly in the liver (Kumar & Robbins, 2009). There are several mediators that act directly on the endothelial cells to increase vascular permeability; these will be discussed a later section.

Chemoattractants

Chemoattractants are soluble molecules that can diffuse away from their site of production and are able to stimulate directional movement of leukocytes (Figure 1A). Endogenous chemoattractants include cytokines, in particular chemokines, components of the complement system and arachidonic acid metabolites. Foreign substances can act as exogenous chemoattractants and the most common are bacterial products. Leukocytes respond to chemoattractants in a process called chemotaxis in which cells move in the direction of increased concentration of a chemoattractant (Franca-Koh & Devreotes, 2004). Examples of chemoattractants that act on neutrophils include the formyl peptide formyl-Methionyl-Leucyl-Phenylalanine (fMLP), complement factor 5a (C5a), leukotriene B_4 (LTB_4) and platelet activating factor (PAF). Neutrophils express receptors (FPR and FPRL1) recognizing fMLP and other formyl peptides from bacteria (Fu *et al.*, 2006). C5a is a product of complement system activation and binds the C5aR and C5L2 receptors on neutrophils (Monk *et al.*, 2007). LTB_4 binds the BLT1 and BLT2 receptors on neutrophils and will be discussed in a later section. Platelet activating factor is a phospholipid-derived mediator, produced by e.g. PMN, EC and platelets, which binds to the PAF receptor on neutrophils (Honda *et al.*, 2002).

Although different chemoattractants are recognized by separate receptors, the receptors share similar features and belong to the pertussis toxin-sensitive subfamily of G protein-coupled receptors (GPCR). Receptor activation will lead to the activation of multiple downstream second messengers and a transient elevation in the intracellular Ca^{2+} concentration resulting in chemotaxis, mobilization of granules, and generation of reactive oxygen species (Fu *et al.*, 2006). Neutrophil exposure to chemoattractants is enhanced during rolling on the endothelium since the contact between the leukocyte and vessel wall is prolonged. The chemoattractants have limited effect in the circulation because they would be rapidly diluted and swept away by blood flow (Springer, 1994).

Cytokines and chemokines

Cytokines are secreted in small amounts in response to external stimuli and bind to high affinity receptors on target cells. Most act in an autocrine or paracrine fashion and generally affect the endothelium to induce adhesion molecule expression and also cause secretion of other cytokines (Figure 1A). The effects are often systemic (Kumar & Robbins, 2009). During systemic infections the amounts produced may be large enough to act in an endocrine manner. Interleukin (IL)-1 and tumor necrosis factor (TNF) are produced by macrophages, EC and some epithelial cells. They cause activation of endothelial cells and leukocytes, act on the hypothalamus to induce fever, and stimulate the liver to synthesize acute phase proteins (Abbas & Lichtman, 2009).

Chemokines are a family of chemoattractant cytokines and include CXC- or α-chemokines (e.g. IL-8) that act on neutrophils and non-hematopoetic cells, and CC- or β-chemokines that affect mononuclear cells (e.g. monocyte chemotactic protein-1, MCP-1, which acts on monocytes) (Charo & Ransohoff, 2006). Chemokines may be displayed to the leukocyte at high concentrations as they can attach to proteoglycans on EC and in the extracellular matrix (Kumar & Robbins, 2009). IL-8 is produced by T lymphocytes, epithelial cells, keratinocytes, fibroblasts, endothelial cells and

neutrophils (Witko-Sarsat *et al.*, 2000). It binds to two receptors (CXCR1 and CXCR2) that are abundant on neutrophils and plays a role in many of the neutrophil antimicrobial functions such as chemotaxis, degranulation and oxidative burst (Stillie *et al.*, 2009).

LEUKOCYTE RECRUITMENT

The recruitment of leukocytes is a key component in inflammatory reactions. Leukocytes are activated at sites of injury or infection and extravasate to the surrounding tissue in a coordinated multistep process that involves rolling along the endothelium, firm adhesion and subsequent migration through the vessel wall (Muller, 2002) (Figure 1A). Recently, additional steps were added to the three step cascade to include slow rolling, adhesion strengthening, intraluminal crawling, paracellular and transcellular migration, and migration though the basement membrane (Ley *et al.*, 2007).

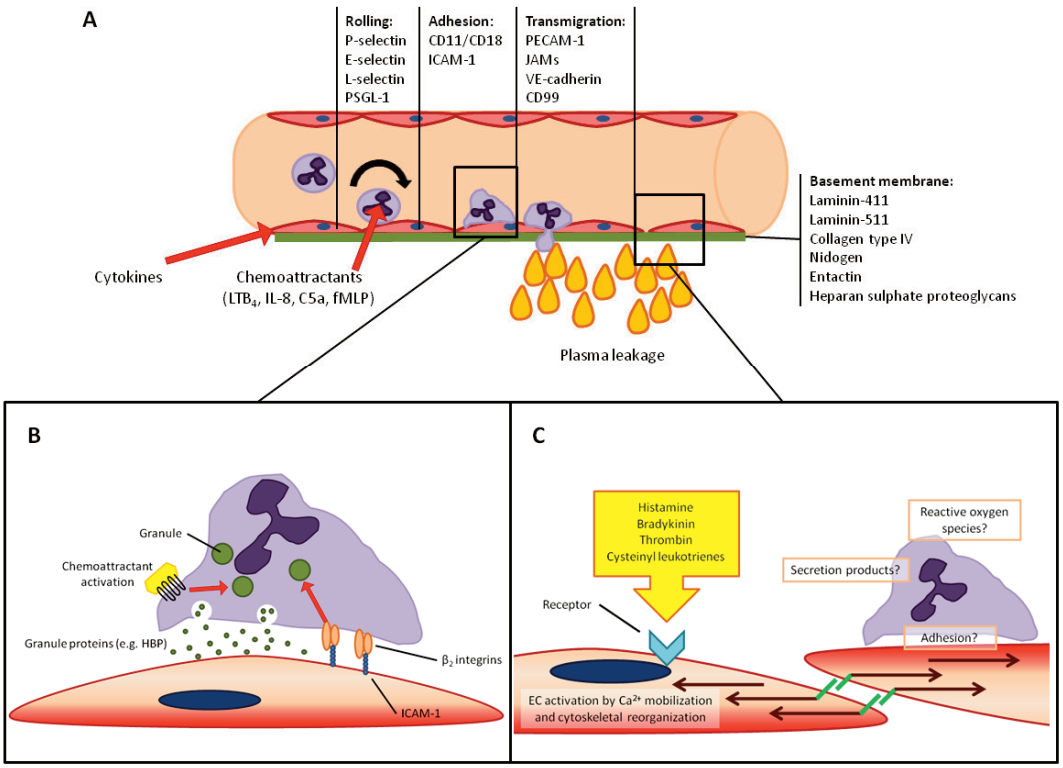

Figure 1. Schematic figure of leukocyte recruitment and plasma extravasation. A) The leukocyte recruitment cascade with key molecules in each step indicated. Cytokines and chemoattractants activate (red arrows) endothelial cells and leukocytes respectively. **B)** Neutrophil granule release as a consequence of β_2 integrin ligation and chemoattractant activation (red arrows) during adhesion to the endothelial cell. **C)** Regulation of vascular permeability by directly acting mediators and possible factors involved in neutrophil-mediated alteration of permeability. Adherens and tight junction proteins, represented in green, associate with the actin cytoskeleton.

Intravascular adhesion

Leukocyte rolling along the endothelium is mediated primarily by the selectins (Ley *et al.*, 2007). L-selectin is constitutively expressed on circulating neutrophils, monocytes and most lymphocytes. P-selectin is stored in the α-granules of platelets and Weibel-Palade bodies in EC and appears on the surface within minutes after stimulation as these granules fuse with the plasma membrane. The expression of E-selectin is limited to endothelial cells (Kansas, 1996). The dominating ligand for all three selectins is PSGL1 (Ley *et al.*, 2007). The interaction between the selectins and their ligands is very loose and the shear stress from the blood causes the leukocyte to detach from the endothelium and bind to another ligand in a repetitive cycle, thus causing the leukocyte to roll along the endothelium. Rolling allows the leukocyte to establish a more stable adhesion to the endothelial surface via integrins (Muller, 2002).

The arrest of leukocytes on the endothelium is rapidly triggered by chemokines or other chemoattractants and is mediated by the ligation between integrins on the leukocytes and immunoglobulin superfamily members such as intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) on the endothelial cells. Cytokines released during inflammation stimulate the EC to express adhesion molecules and synthesize chemokines and lipid chemoattractants that are exposed on the luminal surface of the EC. Chemoattractants are also transported from the abluminal surface and can be generated by proteolytic cleavage in activated macrophages and platelets (Ley *et al.*, 2007). For example, EC produce IL-8 and store it in Weibel-Palade bodies so that it can be rapidly presented on the EC surface (Witko-Sarsat *et al.*, 2000). Many chemokines bind to glycosaminoglycans on the EC surface and this exposure is necessary for leukocyte recruitment (Johnson *et al.*, 2005).

Integrins are heterodimeric receptors that consist of a paired α and β chain and are classified according to the common β chain. There are three major integrin subfamilies among leukocytes; β_1 (CD29), β_2 (CD18), and β_3 (CD61) (Springer, 1994). When leukocytes are stimulated with chemoattractants and chemokines there is a rapid GPCR-signaling which leads to a conformational change of the integrin from a low-affinity to a high-affinity conformation leading to opening of the ligand-binding pocket (Laudanna *et al.*, 2002). In addition to mediating adhesion, integrins generate intracellular signals to regulate functions such as cell motility, proliferation and apoptosis (Ley *et al.*, 2007).

The β_2-integrin CD11/CD18 is expressed exclusively on leukocytes and mediates firm adhesion to vascular endothelium, which is necessary for leukocyte recruitment to extravascular tissue (Arfors *et al.*, 1987). The predominant receptor on the endothelium responsible for CD11/CD18 binding is ICAM-1 (Muller, 2002). There are two types of β_2-integrins that bind to ICAM-1; $\alpha_L\beta_2$ (CD11a/CD18, LFA-1), which is expressed on B and T lymphocytes, monocytes and neutrophils and $\alpha_M\beta_2$ (CD11b/CD18, Mac1), which is expressed on monocytes and neutrophils only (Issekutz & Issekutz, 1992). ICAM-1 appearance on the endothelial cells is increased following stimulation with inflammatory mediators. This induction is largely regulated at the mRNA level and increased surface expression is seen after 4 hours and peaks after 24 hours (Springer, 1990).

Extravasation

Migration through the vessel wall occurs through three barriers: the endothelial cell, the basement membrane and the pericyte sheath which surrounds the vessel. Penetration through the basement membrane takes longer than through the endothelium. Several factors such as chemoattractans, shear flow, and interaction with ICAM-1 and VCAM-1 may stimulate transendothelial migration (Ley *et al.*, 2007). Following adhesion, which is CD11a/CD18 dependent, neutrophils migrate on the endothelial cells to find preferential sites for transmigration in a process called intraluminal crawling and which is mediated by CD11b/CD18 (Phillipson *et al.*, 2006). This process is stimulated by chemoattractants that bind to proteoglycans, e.g. heparan sulfate, on the endothelial cells. Without a chemotactic stimulus which is held in place by proteoglycans, intraluminal crawling, and by extension leukocyte recruitment, is decreased (Massena *et al.*, 2010).

The mechanism of transendothelial migration is not yet completely understood. The prevailing idea is that transmigration is a paracellular process, i.e. the leukocyte migrates through the endothelium by squeezing between adjacent endothelial cells. However, transcellular migration has been suggested as an alternative path through the endothelial lining based on observations in both *in vivo* (Feng *et al.*, 1998; Hoshi & Ushiki, 1999) and *in vitro* models (Carman & Springer, 2004). For example, there is *in vitro* evidence for the formation of "transmigratory cups" rich in ICAM-1, VCAM-1, cytoplasmic proteins and cytoskeletal components that allow leukocytes to migrate through the endothelial cell (Carman & Springer, 2004). However, supposedly this route is only taken by a small percentage of transmigrating cells and it uses similar junctional molecules as during paracellular migration (Ley *et al.*, 2007).

Paracellular migration

Inflamed endothelial cells can redistribute junctional molecules in a fashion that favors transendothelial migration of leukocytes. Several molecules have been identified as key players in paracellular transmigration. Platelet/endothelial cell adhesion molecule-1 (PECAM-1), junctional adhesion molecule (JAM), vascular endothelial cadherin (VE-cadherin) and CD99 have received the most attention. These molecules are localized to the junctions between endothelial cells. They have been investigated both *in vivo* and *in vitro* and have been found to be of different importance in response to varying stimuli and depending on leukocyte subtype.

PECAM-1 is a transmembrane protein, which is expressed by plateles, most subsets of leukocytes, and by endothelial cells. It is concentrated at interendothelial junctions and supports cell-cell adhesion through homotypic interaction (Thompson *et al.*, 2001). Inhibiting the function of this molecule attenuates transendothelial migration *in vitro* (Schenkel *et al.*, 2002) and *in vivo* (Wakelin *et al.*, 1996; Duncan *et al.*, 1999; Thompson *et al.*, 2001). VE-cadherin conjoins adjacent EC through homotypic interaction and is linked to the cytoskeleton and signaling components inside the cell. Distribution of a VE-cadherin antibody *in vivo* enhances neutrophil emigration (Gotsch *et al.*, 1997) indicating that it acts to prevent the passage of leukocytes. In addition, phosphorylation of VE-cadherin, which weakens its adhesive properties, is required for leukocyte transendothelial migration *in vitro* (Allingham *et al.*, 2007;

Alcaide *et al.*, 2008), and transmigration triggers the formation of gaps in VE-cadherin (Alcaide *et al.*, 2008).

As these EC molecules signal to each other through the endothelial cell, it is important that there is a sequential interaction between them and leukocyte (Ley *et al.*, 2007). For example, inhibition of CD99 blocks leukocyte migration distal to PECAM-1 and blockade of both molecules have an additive effect (Schenkel *et al.*, 2002; Lou *et al.*, 2007). There is also evidence that PECAM-1 and JAM-A act sequentially as there is no additive effect of dual blockade (Woodfin *et al.*, 2007). The JAM family is composed of JAM-A to C (the classical members) and the non-classical members endothelial cell selective adhesion molecule (ESAM), coxsackievirus and adenovirus receptor, JAM-4 and JAM-L (Bazzoni, 2003), and has been implicated in the regulation of leukocyte transmigration (Garrido-Urbani *et al.*, 2008).

Migration through the perivascular membrane

Following transendothelial migration, the leukocyte needs to penetrate the perivascular basement membrane (Nourshargh & Marelli-Berg, 2005). The BM is a 50-100 nm thick sheet-like structure that is found basolaterally to the endothelium. It provides structural support and consists of large insoluble molecules that self-assemble via specific binding sites (Kalluri, 2003). The perivascular BM consists of pericytes and extracellular matrix proteins such as laminin-411 and -511 (laminin-8 and -10), collagen type IV, nidogen, entactin and heparan sulphate proteoglycans. The laminins are large heterotrimers consisting of α, β and γ polypeptide chains (Colognato & Yurchenco, 2000). The α4 chain-containing laminin-411 (α4:β1:γ1) is widely expressed in vascular endothelial BM (Iivanainen *et al.*, 1997) and has a significant role in normal blood vessel maturation (Thyboll *et al.*, 2002). Literature covering the field of leukocyte migration through the basement membrane is sparse partly due to difficulties of constructing physiologically relevant *in vitro* models of the basement membrane and associated pericytes. The BM is thought to provide a greater resistance to migration than does the endothelial cell, and there is no consensus as to how the cells migrate through this barrier. Hence, the following mechanisms have been suggested:

1. *Migration through regions with low expression of basement membrane proteins.* Leukocytes have been found to migrate through gaps between pericytes (Wang *et al.*, 2006; Voisin *et al.*, 2010). Pericytes also regulate the presence of low expression sites that are permissive for leukocyte transmigration. These permissive sites are low in collagen IV and laminin-511 and are present in many tissues. The low expression regions are though to act as gates for migrating monocytes and neutrophils (Nourshargh *et al.*, 2010).
2. *Basement membrane remodeling during transmigration*. As PMN migrate through the low expression regions, these regions experience a transient enlargement. The mechanism behind this remodeling is unclear, but evidence exists that neutrophils that have migrated through the BM have laminin fragments on the cell surface indicating possible proteolytic breakdown of laminin (Wang *et al.*, 2006). However, the role for proteases in leukocyte recruitment is inconclusive. There is some support for a role of MMPs (Reichel *et al.*, 2008) and elastase (Young *et al.*, 2004; Wang *et al.*, 2006). On

the other hand, it has been shown that elastase deficiency does not affect leukocyte recruitment to extravascular tissue (Hirche *et al.*, 2004; Wang *et al.*, 2006).

3. *Biochemically or biophysically permissive sites.* The composition of the BM could affect leukocyte transmigration. The laminin α4 chain, but not the α5 chain, is truncated which could lead to reduced cross-linking to collagen IV creating a less dense mesh-work of matrix proteins (Hallmann *et al.*, 2005). This in turn might facilitate migration through the BM. However, there is not yet evidence for this hypothesis.

Following migration through the BM, the leukocyte continues to migrate towards the site of injury or infection through the three-dimensional network of extracellular matrix proteins. The β_1-integrin subfamily includes receptors that bind cells to extracellular matrix components such as fibronectin, laminin and collagen (Springer, 1990; Muller, 2002). Members of the β_1-integrin family are up-regulated as the leukocyte undergoes diapedesis (Werr *et al.*, 1998), and this integrin family has been shown to be primarily responsible for regulating leukocyte migration in the extravascular tissue (Werr *et al.*, 1998; Werr *et al.*, 2000).

PERMEABILITY INCREASES AND EDEMA FORMATION DURING ACUTE INFLAMMATION

The microvascular changes that occur during acute inflammation are designed to maximize the movement of plasma proteins and circulating cells out of the circulation to the site of injury or infection. Following a transient vasoconstriction, there is a dilation of arterioles leading to an increase in blood flow locally as reflected by the heat and redness of the tissue. Several mediators such as prostaglandins, histamine and nitric oxide (NO) acting on smooth muscle cells contribute to the increase in blood flow. The vasodilation is followed by an increased microvascular permeability, leading to leakage of plasma from the vasculature (Kumar & Robbins, 2009) (Figure 1C). A controlled increase in vascular permeability aids in clearing the inflammatory stimulus as the exudate contains complement proteins and antibodies. However, in cases such as influenza-induced pneumonia, burns or brain injury, edema can lead to hospitalization or death (DiStasi & Ley, 2009).

The endothelial barrier and physiologic regulation of vascular permeability

The barrier between the blood and extravascular tissue consists of the endothelium with its glycocalyx and the underlying basement membrane. The resting endothelium acts as a sieve with an average pore size of 3 nm and diffusion of molecules occurs paracellularly (Mehta & Malik, 2006). Although the glycocalyx and basement membrane provide a barrier to macromolecule passage, the primary mechanism behind increased vascular permeability and edema formation in inflammation is endothelial cell contraction leading to paracellular gaps through which plasma macromolecules can pass (Mehta & Malik, 2006).

Fluid exchange across the endothelium is governed by the Starling principle, which states that the net filtration rate depends on the hydrostatic and colloidosmotic

pressures in the intravascular and extravascular compartments. The permeability of the endothelium will also affect the net filtration rate (Boron & Boulpaep, 2005). Inflammatory edema results from an increase in colloid-osmotic pressure in the interstitium as this will draw fluid from the intravascular compartment (Lindbom, 2003). An increase in hydrostatic pressure will augment this effect and the permeability increasing ability of inflammatory mediators such as bradykinin and histamine is enhanced by e.g. prostaglandin E_2 (PGE_2) which acts as a vasodilating agent thus increasing the blood flow to the inflamed tissue (Williams, 1983).

The passage of plasma proteins, solutes and fluid across the barrier created by the endothelium can take two routes – transcellular (via transcytosis) or paracellular (through interendothelial junctions), and endothelial barrier function is most likely regulated by a crosstalk between interendothelial junctions and transcytosis. Transcellular passage occurs primarily through vesicular transport. This mechanism is initiated by fission of caveolin-1 rich domains of plasma membrane, caveolae, on the luminal side of the EC. The caveolar vesicles are then transported to the basal surface where they fuse with the plasma membrane and release macromolecules by exocytosis (Komarova & Malik, 2010). The permeability increase during inflammation is however most likely through the paracellular route.

Paracellular permeability is regulated by the interplay of adhesive forces between the endothelial cells and the counteradhesive forces generated by endothelial actomyosin contraction (Mehta & Malik, 2006). Endothelial cells are held together by adherens junctions (AJ) and tight junctions (TJ) that have comparable functions although they are formed by different molecules. Similar between the two is that adhesion is mediated by transmembrane proteins that promote homophilic interaction between adjacent cells. AJs are made up of a complex consisting of VE-cadherin, catenins and plakoglobin (Dejana, 2004). Five cadherin like repeats make up VE-cadherin that associates with VE-cadherin on adjacent EC in a Ca^{2+} dependent manner. Intracellularly, VE-cadherin binds p120-catenin (p120), and α- and β-catenins, which link VE-cadherin to the cytoskeleton (Vandenbroucke *et al.*, 2008). The interactions between cadherins, kinases and the Rho GTPases are regulated by p120 which controls the phosphorylation and stability of cadherin-cadherin and cadherin-catenin interaction (Mehta & Malik, 2006). Tyrosine phosporylation of VE-cadherin and other components of AJs are associated with weak junctions and impaired barrier function (Dejana *et al.*, 2008), and an antibody against VE-cadherin results in increased vascular permeability *in vivo* (Corada *et al.*, 1999). Vascular endothelial protein tyrosine phosphatase (VE-PTP) supports VE-cadherin function and is required for maintaining EC barrier function (Nottebaum *et al.*, 2008). Tight junctions mediate adhesion between cells by members of the claudin family, occludin, JAMs A-C and ESAM (Dejana, 2004). The composition of TJs and AJs differ depending on the vascular bed. AJs, consisting of VE-cadherin mediated adhesion, are thought to maintain structural integrity of the junctions whereas TJs are secondary and appear mainly in larger blood vessels and as part of the blood-brain barrier (BBB, see later section) (Komarova & Malik, 2010).

Contraction of vascular EC is regulated by actin-myosin interaction. This interaction is mainly controlled by phosphorylation of myosin light chain (MLC), which can be altered by three different mechanisms: 1) direct phosphorylation by myosin light chain kinase (MLCK); 2) dephosphorylation of MLC by myosin light chain phosphatase (MLCP), and 3) inhibition of MLCP by RhoA through its downstream

effector Rho kinase (ROCK) thus potentiating MLC phosphorylation (Vandenbroucke *et al.*, 2008). MLC phosphorylation is indicated in EC contraction by several mediators such as bradykinin, histamine, thrombin and in response to neutrophil activation, and inhibition of MLCK attenuates increases in vascular permeability (Yuan, 2002). Phosphorylation of MLC is Ca^{2+} dependent and the disruption of AJs through microtubule destabilization, which results in EC contraction occurs in a RhoA/ROCK dependent manner (Vandenbroucke *et al.*, 2008).

The family of small Rho GTPases, including RhoA, Cdc42 and Rac1, are key regulators of actin reorganization and signaling in endothelial cells (Strey *et al.*, 2002). It has been shown that Rac1 and Cdc42 signaling is important in stabilizing the endothelial barrier (Wojciak-Stothard *et al.*, 2005). RhoA and ROCK, on the other hand, have been assigned an excitatory role in hyperpermeability caused by histamine or PMN activation (Wojciak-Stothard *et al.*, 2001; Breslin & Yuan, 2004). Rac1 acts upstream of RhoA and prevents endothelial cell contraction. When Rac1 is inhibited, as during endothelial cell hypoxia, RhoA is activated leading to stress fiber formation (Wojciak-Stothard *et al.*, 2005).

The endothelium is the major barrier to macromolecules. However, the glycocalyx on endothelial cells and the basement membrane also contribute to the barrier function. The glycocalyx, which is a negatively charged surface coat of proteoglycans, glycosaminoglycans and plasma proteins lining the luminal side of the endothelium, may limit the passage of macromolecules, especially charge-selectively, to the endothelial cell surface (Mehta & Malik, 2006). The glycocalyx shields the vascular wall from direct exposure of blood flow by its positioning at the interface between the blood and the endothelium. It has been implicated in the maintenance of endothelial permeability and it is suggested that the protein concentration gradient which is necessary for colloid osmotic pressure is localized across the glycocalyx (van den Berg *et al.*, 2006). Degradation of endothelial glycocalyx in the myocardium leads to edema formation (van den Berg *et al.*, 2003). The basement membrane could add to the barrier function of the endothelium by interaction between its components and integrins on the endothelial cell leading to enhanced cell-cell adhesion (Mehta & Malik, 2006).

Sphingosine 1-phosphate has been shown to enhance EC barrier function both *in vivo* and *in vitro*. It is released from erythrocytes and platelets and binds to receptors on the endothelium where it causes increased VE-cadherin and β-catenin expression resulting in enhanced barrier function (Wang & Dudek, 2009). Other molecules that are able to enhance barrier function are activated protein C and adenosine, which can do so through several mechanisms (DiStasi & Ley, 2009).

Directly acting mediators of increased vascular permeability

Many soluble factors of different origin such as thrombin, bradykinin, histamine, cysteinyl leukotrienes, oxygen free radicals, and vascular endothelial growth factor (VEGF) are known to induce increased vascular permeability through direct action on the endothelial cells (Mehta & Malik, 2006; Vandenbroucke *et al.*, 2008) (Figure 1C).

Leukotrienes and prostaglandins

Prostaglandins and leukotrienes (LT) are arachidonic acid derivatives that have long been known to mediate inflammatory responses such as vasodilation, increased vascular permeability and neutrophil recruitment. Prostaglandins can be formed by most cells and LT are predominantly made by inflammatory cells such as PMN, macrophages and mast cells (Funk, 2001). 5-LO is the key enzyme in the cascade, which transforms arachidonic acid to LTA_4. LTA_4 can be hydrolyzed yielding LTB_4, or conjoined with glutathione to form LTC_4. LTC_4 can in turn be metabolized extracellularly yielding LTD_4 and LTE_4. A collective name for LTC_4, LTD_4 and LTE_4 is the cysteinyl leukotrienes and they are known for their slow and sustained smooth muscle contracting abilities. LTB_4 is a potent neutrophil chemoattractant thus stimulating neutrophil recruitment (Funk, 2001). Leukotrienes act at GPCRs. Leukocytes express the BLT_1 and BLT_2 receptors which bind LTB_4 with high and low affinity, respectively. These receptors cause neutrophil activation leading to chemotaxis and degranulation (Tager & Luster, 2003). The cysteinyl leukotrienes bind to the Cys-LT_1 and Cys-LT_2 receptors that are present on airway smooth muscle cells and vascular endothelial cells (Funk, 2001), and are known as potent inducers of increased vascular permeability (Dahlen *et al.*, 1981).

Histamine

Histamine is stored preformed in mast cell granules and can therefore be released quickly. Mast cells are localized in the connective tissue adjacent to blood vessels and release histamine as a response to several stimuli such as allergic reactions, anaphylatoxins, substance P and cytokines (Kumar & Robbins, 2009). The permeability increasing effect of histamine results from activation of the H_1 receptor on endothelial cells (Repka-Ramirez & Baraniuk, 2002). Histamine also acts as a vasodilator, predominantly via the H_2 receptor, thus enhancing edema formation.

Bradykinin

A distinct chemical entity with established impact on vascular permeability is represented by the kallikrein/kinin system, known also as the contact phase system, with its major components high molecular weight kininogen (HK), plasma kallikrein (PK), and factor XII (FXII). These proteins assemble on the surface of endothelial cells and of PMN (Schmaier, 2008). Kinins, with bradykinin being its best characterized member, are low-molecular weight peptides that have the ability to activate endothelial cells and cause vasodilation, increased vascular permeability, production of NO and arachidonic acid mobilization. They are also able to stimulate sensory nerve endings causing pain. These effects make them an important player in the inflammatory process as they are able to elicit the cardinal signs of inflammation. Bradykinin can be generated via two general pathways:

1. Tissue kallikrein, which is secreted by many cells in the body, digests low-molecular weight kininogen (LK) to yield kallidin that is cleaved to form bradykinin.

2. The second mechanism for bradykinin formation involves molecules of the intrinsic coagulation pathway. The initiating protein in this cascade is Factor XII, which binds to negatively charged macromolecular surfaces and autoactivates to generate FXIIa. Factor XIIa acts on plasma prekallikrein which circulates as a complex with HK. The PK-HK complex binds to endothelial cells with the HK domains 3 and 5. Activation of plasma prekallikrein by Factor XIIa leads to the formation of kallikrein, which is able to cleave HK to generate bradykinin (Kaplan *et al.*, 2002).

Bradykinin binds to two types of receptors on the endothelial cell, B1 and B2. The B2 receptor is constitutively expressed and the B1 receptor is induced by inflammatory stimuli such as TNF and IL-1. The kinin receptors are GPCRs and activate several second messenger systems leading to increased Ca^{2+} and activation of protein kinase C (PKC) (Howl & Payne, 2003). In vitro studies activating the kallikrein-kinin system have generally been performed with non-physiological negatively charged surfaces such as glass or kaolin, and generation of bradykinin in the contact of blood with these surfaces explains the alternative name "the contact system". The physiological activator of the system is yet unknown (Kaplan *et al.*, 2002).

Thrombin

Thrombin is a major product of the coagulation cascade. Proteolytic cleavage of prothrombin results in the formation of thrombin which binds to three different proteinase-activated receptors (PARs). PAR-1 is located on endothelial cells and activation of this receptor results in increased vascular permeability (Bogatcheva *et al.*, 2002).

Neutrophil-induced permeability increases in acute inflammation

It has been known for long that PMN induce increased vascular permeability. Wedmore and Williams showed, in 1981, that PMN are required for the permeability increase induced by chemoattractants such as fMLP, C5a, and LTB_4 (Wedmore & Williams, 1981). The mechanism with which neutrophils cause edema formation is yet unknown, and there are several ways that PMN can stimulate alterations in vascular permeability; via secreted products, via adhesion, via transmigration or by the release of ROS (Wang & Doerschuk, 2002) (Figure 1C).

Neutrophil adhesion as a mechanism for increased permeability

It was previously thought that the PMN-associated increase in permeability were due to leakage of plasma during PMN transendothelial migration (Lindbom, 2003). However, adhesion alone can induce this phenomenon (Gautam *et al.*, 1998; Lindbom, 2003). That PMN do not have to migrate through the endothelium for edema formation to occur is supported by the rapid response in permeability alteration. When PMN attach there is an increase in Ca^{2+}, MLCK activation and actin cytoskeleton remodeling leading to increased permeability (Huang *et al.*, 1993). This occurs concurrently with signaling to the endothelial cell junctional proteins that become

phosphorylated and there is a downregulation of the adhesive contacts between the endothelial cells (Bolton *et al.*, 1998; Tinsley *et al.*, 1999).

Neutrophils induce MLC phosphorylation and activation of RhoA, ROCK and fokal adhesion kinase which leads to stress fiber formation, and dissociation of EC junctional proteins resulting in gap formation (Lindbom, 2003). As the PMN adheres, primarily through β_2-integrin binding to ICAM-1, there is a rapid and transient increase in endothelial $[Ca^{2+}]_i$ (Gautam *et al.*, 1998). This results in cytoskeletal changes caused by increased interaction between actin and myosin light chain, and oxidant production in the endothelial cell (Wang & Doerschuk, 2002). Blockade of the Ca^{2+} mobilization (Huang *et al.*, 1993; Gautam *et al.*, 2000) or MLC phosphorylation (Yuan *et al.*, 2002) prevents the PMN-induced permeability increase.

It has been shown, using *in vitro* models, that blockade of ICAM-1 inhibits these cytoskeletal changes and that cross-linking of ICAM-1 is able to activate several signaling molecules (such as PKC, Rho, Src family kinases and Ca^{2+}) that could lead to cytoskeletal reorganization (Wang & Doerschuk, 2002). Engagement of ICAM-1 leads to activation of the tyrosine kinases Src and Pyk2, which is required for phosphorylation of VE-cadherin, which in turn is required for PMN transmigration (Allingham *et al.*, 2007). PMN binding to TNFα-activated EC initiates the dissociation of VE-PTP from VE-cadherin which weakens the VE-cadherin function. This dissociation was independent of EC adhesion molecules or oxygen free radicals (Nottebaum *et al.*, 2008).

Neutrophil-secretion as a mechanism for increased permeability

Outside-in signaling by β_2-integrins triggers the secretion of PMN-derived factors that cause permeability increases independent of activation of endothelial receptors (Gautam *et al.*, 2000). As described earlier, PMN contain three types of granules as wells as secretory vesicles that are released in a timely fashion during the recruitment process (Borregaard *et al.*, 2007). Several of the granule proteins such as neutrophil elastase, cathepsin G and HBP have been shown to be capable of increasing vascular permeability (DiStasi & Ley, 2009).

Neutrophil elastase and cathepsin G are present on the cell surface of transmigrating PMN in vitro and are able to cleave VE cadherin thus disrupting the EC monolayer (Hermant *et al.*, 2003). Membrane bound elastase will be protected from protease inhibitors in the plasma, which allows for targeting of the enzymatic activity to junctions where transmigration occurs. Elastase has been found to be localized to the leading front of transmigrating cells (Cepinskas *et al.*, 1999). On the contrary, mice that are deficient in elastase exhibit, despite an attenuation of the number of recruited PMN, an increase in permeability (Kaynar *et al.*, 2008) possibly due to longer adhesion time at the endothelium. In an *in vitro* model of the endothelial barrier, selective removal of elastase and cathepsin G from supernatants of activated PMN had no effect on the permeability increasing activity of the PMN supernatants (Gautam *et al.*, 2001). The role for elastase in PMN-induced permeability increase is disputed also by the time span and prestimulation required for increased permeability to occur (Smedly *et al.*, 1986), presumably due to the slow mobilization of elastase from the primary granules. Further, it is not clear if adherent or transmigrating PMN

release granules beyond secretory vesicles (DiStasi & Ley, 2009).Few studies have investigated the role of elastase and cathepsin G *in vivo*.

Opposed to what has been suggested for elastase and cathepsin G, neutrophil granule proteins may not act to increase permeability using a proteolytic effect but rather via charge interactions (Peterson *et al.*, 1987; Peterson, 1989; Rosengren & Arfors, 1990, 1991; Gautam *et al.*, 2001). The inactive serine protease family member HBP, which is released from PMN upon β_2 ligation (Figure 1B), has also been suggested as a link in PMN-induced alterations of vascular permeability (Gautam *et al.*, 2001). It is stored in the primary granules together with elastase, cathepsin G and proteinase 3, but a substantial part is located also in the secretory vesicles and can therefore be mobilized more rapidly than the other serine proteases (Tapper *et al.*, 2002). HBP binds to surface proteoglycans on the EC, probably via its concentration of strong positively charged aminoacids that creates a strong dipole moment (Gautam *et al.*, 2001). However, the mechanism behind charge dependent increases in permeability remains unknown.

Reactive oxygen species-induced mechanisms of vascular permeability.

Neutrophils are able to produce highly reactive oxygen species through their granule contents and although these are intended for microbicidal effects in the tissue and should not be released in the blood stream it is possible that these ROS contribute to alterations in permeability (Dallegri & Ottonello, 1997). ROS may cause increased permeability either via direct damage to the endothelial cell, or activation of signaling pathways leading to increased intracellular Ca^{2+}, MLCK activation and cytoskeletal reorganization (Lindbom, 2003).

As these products are highly reactive and thus potentially harmful to the tissue it seems unphysiological for them to be released at the endothelium to increase permeability. Still there is evidence to support that ROS may cause disruption of the endothelial barrier through cell disintegration, increased Ca^{2+}, MLCK activation and junctional protein reorganization (Lindbom, 2003). In addition, endothelial cells are able to produce these ROS as a consequence of PMN activation (Wang & Doerschuk, 2002). However, there is evidence to dispute the role of PMN-induced ROS formation leading to increased vascular permeability (Harlan *et al.*, 1985; Rosengren *et al.*, 1988; Kaslovsky *et al.*, 1990). Oxidants have been shown to stimulate endothelial cells to express PMN adhesion molecules and this may be an indirect reason for ROS-induced alterations in endothelial barrier function (Lo *et al.*, 1993). Additionally, clinical trials with antioxidant therapy to prevent edema formation have been unsuccessful (Boueiz & Hassoun, 2009).

THE BLOOD-BRAIN BARRIER AND FORMATION OF CEREBRAL EDEMA

As already mentioned, the BBB includes a specialized endothelium limited to vessels of the central nervous system (CNS). The endothelial junctions are structurally different from those in the peripheral endothelium and therefore the formation of cerebral edema requires special attention.

The Blood Brain Barrier

Through a complex network of tight junctions, the endothelium making up the BBB restricts diffusion of water-soluble molecules across the vessel wall. Pericytes surround endothelial cells with their pseudopodia and support the barrier function of the endothelium. The BBB protects the brain from noxious stimuli by tight control of trans- and paracellular transportation processes. Intact BBB does not easily let through blood components larger than 20 kDa and the transport of substances into the brain depends on the size and lipid solubility of the molecule as well as the presence of specific carrier systems (Scholz *et al.*, 2007). Maintenance of ion homeostasis is crucial because of its importance for neuronal function and as proteins such as albumin and plasminogen are damaging to nervous tissue, it is crucial that the BBB prevents macromolecule leakage from the blood (Abbott *et al.*, 2010).

The BBB consists of three layers: endothelium, basal membrane, and astrocyte pseudopodia. Microvessel endothelial cell association with astrocytic glia endfeet, contributes to the specialized BBB phenotype. The morphology, biochemistry and function distinguish these EC from those making up the endothelial lining in peripheral tissue (Engelhardt & Sorokin, 2009). In addition to the adherens junctions that promote endothelial barrier function in the peripheral tissue, the BBB has well developed tight junctions that are responsible for the restriction of diffusion (Wolburg *et al.*, 2009). This junctional complex consists of occludins and claudins that span the intercellular cleft together with JAM. The regulatory proteins zonula occludens (ZO)-1 to -3 and cingulin link occludin and claudin to the cytoskeleton. Dysfunction of the BBB is associated with several CNS pathologies such as multiple sclerosis, viral and bacterial infections, skull trauma and stroke (Abbott *et al.*, 2010).

Cerebral edema

Brain edema is a result of structural and functional changes of the BBB, microcirculation, and cell volume regulation. Edema can be classified as cytotoxic or vasogenic. Two additional categories of brain edema include interstitial edema seen in patients with hydrocephalus and “osmotic” edema caused by electrolyte imbalances leading to water influx into cells (Unterberg *et al.*, 2004). Vasogenic edema results from extracellular water accumulation due to loss of BBB integrity or disturbed microcirculation, and is characterized by normal cell size and increased interstitial space. The BBB dysfunction could be the effect of mechanical injury or inflammatory mediators or a combination of both. Paracellular permeability is the major pathway for plasma leakage across the BBB (Abbott *et al.*, 2010) and disruption of the BBB is characterized by intracellular gap formation, changes in cell shape, cytoskeletal reorganization and redistribution of endothelial junctional proteins (Stamatovic *et al.*, 2006).

Cytotoxic edema occurs as a result of intracellular swelling of glia and neurons because of water accumulation due to cellular injury. This form of edema may arise independently of the integrity of the BBB and is characterized by cell swelling and decreased interstitial space (Unterberg *et al.*, 2004). Osmotic/interstitial brain edema is usually caused by serum hypoosmolarity but can also be the result of hyperosmolarity of the cerebral tissue as seen following ischemia or traumatic brain injury (TBI) where necrotic tissue can have high osmolality. Traditionally, cerebral edema following TBI has been considered as vasogenic, especially around contusions. It is now known that cytotoxic edema also is of significant importance (Unterberg *et al.*, 2004).

Inflammation and brain edema – role for neutrophils?

The CNS has historically been thought to be an immunologically privileged organ because of the tight blood brain barrier. However, more recent research has shown that several cell-types such as neurons, astrocytes and microglia are able to synthesize immune mediators and it is now accepted that injury to the brain can elicit a potent immune response (Schmidt *et al.*, 2005). The barrier function can be modified by inflammatory events such as leukocyte activation and release of free radicals and inflammatory mediators like histamine, bradykinin, TNFα or IL-1, that are also known to affect vascular permeability in peripheral tissue (Scholz *et al.*, 2007; Abbott *et al.*, 2010). Proinflammatory cytokines and oxidative mediators participate in BBB disruption either directly or via other mediators thus causing brain edema (Stamatovic *et al.*, 2006). In the brain, the endothelium is mainly activated by local release of cytokines from parenchymal or vascular cells (Scholz *et al.*, 2007). The neuroinflammatory response includes activation of gial cells, intrathecal release of proinflammatory cytokines and chemokines, upregulation of EC adhesion molecules and intracranial complement activation (Schmidt *et al.*, 2004).

Neutrophils are able to enter the brain through transendothelial migration across the BBB or by direct migration from the blood stream during a hemorrhage (Joice *et al.*, 2009). The ability of neutrophils to exacerbate injury has been shown in several CNS conditions (Bednar *et al.*, 1997). During the first 24 hours following TBI, there is a recruitment of PMN into the brain parenchyma and possibly a relationship between cortical PMN accumulation and secondary brain injury (Zhuang *et al.*, 1993). Activation of PMN is tightly associated with the production and secretion of cytokines/chemokines and the release of oxygen radicals and proteases (Scholz *et al.*, 2007).

Leukocyte recruitment across the BBB is associated with the activation of signaling cascades leading to the loss of TJ proteins occludin and ZO-1 and redistribution of AJ proteins (Bolton *et al.*, 1998). This may explain the significant correlation between PMN accumulation and brain edema (Schoettle *et al.*, 1990). In addition, the CXC chemokine IL-8, which causes PMN recruitment, is associated with BBB dysfunction (Kossmann *et al.*, 1997) and clinical outcome following TBI (Whalen *et al.*, 2000). However, whether there is a causal relationship between the two remains to be determined as there is evidence both for (Schoettle *et al.*, 1990) and against (Whalen *et al.*, 1999) a role of PMN in cerebral edema formation.

AIMS

The overall aim of this thesis was to further elucidate mechanisms controlling leukocyte extravasation and concurrent alterations in vascular permeability in acute inflammation. More specifically, the studies aimed at investigating:

1. The importance of the basement membrane protein laminin-411 in leukocyte recruitment to extravascular tissue.

2. The mechanistic basis behind neutrophil-induced alteration of vascular permeability.

3. The role of neutrophils in the edema formation following traumatic brain injury.

EXPERIMENTAL PROCEDURES

The studies included in this thesis are based on a combined use of *in vivo* models and *in vitro* methodology adapted to the specific research questions. For a more detailed description of the methods used, see the individual papers.

IN VIVO METHODOLOGY

As the immune system of animals is broadly similar across species and the inflammatory mediators in humans usually have homologous counterparts in rodents, the use of rodent models to study inflammation is invaluable (Moore, 2003). A multitude of *in vivo* models have been developed to study the inflammatory process. These range from simple screening-type models to more complex models such as intravital microscopy of exposed tissues.

Inflammation of the ear skin (paper I)

Inflammation of the ear skin is a well established and commonly used model, where an inflammatory stimulus is applied to one ear using the other ear as a control. Several different substances can be used to induce inflammation (e.g. croton oil, mustard oil and zymosan) and ear thickness or weight is used as a measure of the inflammatory response (Gabor, 2003). Results are often expressed as a ratio between the inflamed and control ear. In paper I, croton oil application to the right ear was used as a stimulus to induce an acute inflammatory reaction. The left ear served as a control. Mice were sacrificed five hours later and ear pieces were removed using a dermal punch, and weighed.

A delayed time hypersensitivity (DTH) reaction was also induced in the ear skin. In this model mice were sensitized through application of 1-Fluoro-2,4-dinitrobenzene (DNFB) on the rear foot pad for two days. Four days later the mice were challenged by application of DNFB to the right ear and measurements were performed after 24 hours. In contrast to croton oil-induced inflammation, which is a crude model resulting in recruitment of several leukocyte subclasses, lymphocytes constitute the predominant cell type infiltrating the tissue in response to DNFB (Phanuphak *et al.*, 1974).

Both ear models are quick and simple, require small quantities of substances and provide well-reproducible results. However, without histological examination of the ears, the composition of the exudate, which causes increased ear weight and thickness, cannot be specified. Further examinations using models that allow for such analysis should therefore be performed as a complement.

Cavity models (paper I, II and III)

Slightly more complex techniques than the ear skin model are represented by different cavity models, such as the subcutaneous air pouch, peritonitis and pleurisy. In these models, an inflammatory stimulus is injected into a physiological or artificial cavity and the exudate is harvested and analyzed (Moore, 2003). In this thesis, the subcutaneous air pouch and pleurisy models were used, and as they have different characteristics they can be used in combination to achieve a better understanding of the complex inflammatory process.

The air pouch model involves the creation of a subcutaneous pocket on the back of the mouse. Air is injected twice over 4-6 days and the inflammatory substance is then injected and exudate can be harvested at several time points later (Colville-Nash & Lawrence, 2003). One advantage of this model is that as the air pouch is an artificial cavity so the number of resident cells that interfere with the inflammatory response are few. This model also allows for the use of a multitude of stimuli and treatment times. Finally, it is minimally invasive and many mice can be treated simultaneously. In paper I, MCP-1 was injected into the pouch to stimulate recruitment of monocytes specifically. Mice were sacrificed 24 hours after injection, the pouch was lavaged and the collected cells were differentiated using flow cytometry.

Pleurisy is a more advanced cavity model as it allows for parallel assessment of vascular permeability and leukocyte recruitment. Induction of pleurisy is performed through direct injection of the irritant to the pleural cavity in anaesthetized mice or rats. A fluorescently labeled macromolecule is administered intravenously as a plasma marker and following sacrifice, the pleural exudate is collected, and its volume measured and analyzed for leukocyte and macromolecule content. The most common models of pleurisy use carrageenan as the inflammatory stimulus (Moore, 2003). We found however that the permeability increase using this irritant was not solely PMN-dependent and since this was important to test our hypothesis we used thioglycollate instead (paper I and III). Thioglycollate is known to become more potent over time (Li *et al.*, 1997), and therefore control experiments were always performed together with the treatments. In paper II, pleurisy was induced using LTB_4.

Prior to surgical intervention, mice were anaesthetized and a jugular vein catheter was placed for intravenous access. As this model requires invasive surgery, the mice were kept anaesthetized during the whole experiment. FITC-dextran was used as a plasma macromolecule tracer. Mice were sacrificed four hours after the intrapleural injection of stimulant. A blood sample was taken to determine the serum level of fluorescein isothiocyanate (FITC)-dextran and the exudate in the pleural cavity was removed for analysis of macromolecule and leukocyte content.

Intravital microscopy (paper I and III)

Intravital microscopy (IVM) allows for a more thorough investigation of leukocyte recruitment and alteration of vascular permeability than previously described models. The investigated tissue in an anaesthetized animal is exteriorized and observed with a microscope. Using a camera and recording of images, it is possible to analyze microcirculatory parameters such as leukocyte behavior and permeability changes in real time. IVM provides information about the different steps in the leukocyte extravasation cascade (rolling, adhesion, transmigration and extravascular migration).

Chemoattractants can be applied in several manners; superfusion, interstitial microinjection, intravenous injection, or by placing a gel containing a chemoattractant at a specific distance from the venule (Cara & Kubes, 2004). Vascular permeability changes can be investigated by intravenous injection of a fluorescent plasma marker (Raud & Lindbom, 1994). The method has previously been limited to tissues that can be easily transilluminated, e.g. cremaster muscle and mesentery in mouse and rat, and the hamster cheek pouch. However, as the method has been further developed it is now possible, using fluorescent microscopy, to study tissues such as the brain, liver, large arteries and heart (Cara & Kubes, 2004). In this thesis IVM of the mouse cremaster muscle (paper I) and hamster cheek pouch (paper III) was used.

The mouse cremaster is commonly used for assessment of leukocyte recruitment. Rolling cells are classified as those moving slower than red blood cells, adherent cells as those remaining stationary for a set amount of time and extravasated cells are counted in a predetermined distance from a post-capillary venule. Extravascular migration can be assessed using transillumination and time-lapse video microscopy. Due to the skeletal muscle striations, it is easy to detect extravasated cells using this model. In paper I, PAF was added to the superfusion to stimulate leukocyte recruitment and the number of adherent and extravascular cells was counted before and after a 60 min period. For assessment of extravascular migration velocity, 60 min recordings were made with time-lapse video microscopy and the distances traveled by leukocytes were analyzed off-line.

The hamster cheek pouch was used, in paper III, to simultaneously assess changes in vascular permeability and leukocyte adhesion, which was stimulated with the addition of LTB_4 to the superfusion solution. Leukocyte independent plasma leakage was induced by topical administration of bradykinin. Vascular permeability was assessed using an intravenous injection of FITC-dextran, and transillumination with excitatory fluorescent light. Quantification of leakage from the vessels can be performed using several methods. The leakage sites can be counted under low magnification. Another possibility is to collect the superfusion fluid and measure its fluorescent content (Raud & Lindbom, 1994). In our experiments, we used photodensitometric determination of images taken. A drawback to this method of measuring permeability is that is does not measure the inflammatory response of a whole tissue. On the other hand, it allows for detailed temporal and spatial determination of leakage sites.

It is important to maintain physiological conditions by minimizing the "unspecific" inflammation resulting from preparative surgery and establishing a physiologic environment around the preparation with respect to temperature, extracellular fluid composition, pH and gas tension (Raud & Lindbom, 1994). Blood flow should also be controlled as this might affect leukocyte function and vascular permeability. We found that PGE_2 should be added to the superfusion for optimal blood flow in the hamster cheek pouch. There is a fine line between preparing the tissue well enough to obtain good images and on the other hand causing damage to the tissue, which may affect both leukocyte behavior and vascular permeability. Leukocyte rolling as a consequence of the preparative procedure is very difficult to prevent, but adhesion and extravasated cells should be minimal following surgical preparation. A major drawback to intravital microscopy is that it is time-consuming because of delicate surgery and that the technique allows study of only one animal at a time. There is also a risk for bias when choosing the locations to be measured. We therefore analyzed 4-6

pre-determined areas in each preparation and followed them throughout the experiment.

Traumatic brain injury (paper IV)

TBI is a multifaceted condition and no single experimental technique can reproduce all of its characteristics. A major challenge with trying to model TBI is that real-life injury may vary with respect to location, cause and severity. Therefore, several different models are available out of which the most common are the weight drop, impact acceleration, controlled cortical impact (CCI) and fluid percussion models. Except for the impact acceleration model, which results in a diffuse injury, these models cause focal brain injury (Morales *et al.*, 2005).

The CCI model was first described in the rat by Dixon et. al. (Dixon *et al.*, 1991) and uses mechanical energy to induce damage. It uses a pneumatically driven piston, which impacts a localized area of the exposed dura. This model results in less mortality and apnea than the fluid percussion model due to decreased involvement of the brain stem (Dixon *et al.*, 1991). The site of injury can be either along the midline or lateral cortex. The decrease in mortality compared to other models can be both of advantage and disadvantage depending on the hypothesis being tested. A strong positive side to this model is the ability to control the severity of the injury by altering the time, velocity and depth of the impact. Also, with the same operator, the extent of injury is very reproducible. The damage caused by CCI is predominantly focal and has been shown to cause acute subdural hematoma, axonal injury, concussion, BBB dysfunction and coma (Morales *et al.*, 2005).

In paper IV, CCI in mice was used as a model of TBI. Mice were anesthetized and cortical contusion was delivered by a piston to the right hemisphere. Depending on the outcome measurement, mice were sacrificed 24 hours to 14 days later. To determine lesion size, sections of right and left hemispheres were stained and photographed. Immunohistochemistry and antibody staining was used to assess parenchymal cell apoptosis and microglial activation. Two methods were used to determine edema formation; brain water content and Evan's blue extravasation. Brains were weighed directly after removal and again after drying to assess water content. Evan's blue, which binds to albumin in the blood, was injected intravenously via the tail vein after CCI. Extravasation of the dye after injury was assessed spectrophotometrically following extraction from brain tissue in formamide.

IN VITRO METHODOLOGY

Although inflammation, with characteristical recruitment of leukocytes and increase in vascular permeability, is a complex process hard to model using *in vitro* systems, the importance of such techniques should not be overlooked. They allow for direct observation, quantitative analysis and manipulation of inflammatory parameters that are currently not possible to track *in vivo* (Muller & Luscinskas, 2008). Most *in vitro* model systems focusing on the inflammatory reaction take advantage of leukocytes isolated from whole blood and cultured endothelial cells.

Endothelial cell culture (paper II and III)

In vitro, EC monolayers have been widely used to model the vessel wall and to study adhesive interactions of immune cells in the microvasculature. Although using cultured EC to model inflammatory reactions is convenient and versatile, there are some limitations to their use. For example, EC from different organs and/or species behave differently in culture. Furthermore, there might be a difference in the behavior of micro- and macrovascular EC, and because inflammation generally takes place in the microcirculation, this difference may be of importance (Kvietys & Granger, 1997). *In vivo*, endothelial cells are constantly exposed to shear stress and EC that are cultured under shear have been shown to express more developed adherens and tight junctions (Noria *et al.*, 1999; Colgan *et al.*, 2007). However, the role of shear stress in EC culture is yet to be fully understood (Tarbell, 2010).

Human umbilical vein endothelial cells (HUVEC) were used in paper II and III. In paper II, Ca^{2+} mobilization in HUVEC was assessed using the Calcium indicator FURA-2AM., In paper III, HUVEC were used to investigate proteolysis of HK induced by PMN. HUVEC were incubated with human plasma and neutrophils were then added and activated with IL-8. Western blotting was used to detect intact HK in cell lysates.

Human neutrophils (paper I, II and III)

Neutrophils from human venous blood were used in paper I, II and III. Cells were isolated using conventional separation media and red blood cells were subjected to hypotonic lysis. In paper I, neutrophil migration on laminin-411 or -511 was induced by IL-8 in a modified Boyden chamber chemotaxis assay. Migrated cells were collected and analyzed by flow cytometry. In another set of experiments neutrophil adhesion to laminin-411 and -511 was stimulated using IL-8. Adherent cells were quantified through analysis of MPO activity (Suzuki *et al.*, 1983). In paper II, isolated PMN were stimulated with LTB_4 to induce degranulation. Cells were spun down and the supernatant, containing neutrophil granule proteins, were used to stimulate endothelial cells *in vitro* and to induce vascular permeability *in vivo*.

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RESULTS AND DISCUSSION

The cardinal signs of inflammation have been known for two thousand years and much research has been performed to clarify the mechanisms that lead to the recruitment of leukocytes and alterations in vascular permeability. However, many details remain unclear. With the use of *in vivo* techniques and *in vitro* methodology, this thesis aimed at further elucidating the interplay between emigrating leukocytes and the vessel wall in acute inflammation.

Inflammatory cell migration across the vessel wall is dependent on the presence of laminin α4 in the basement membrane (paper I)

Leukocyte recruitment in inflammation involves migration across the endothelium and the perivascular basement membrane, which consists of a dense network of matrix proteins such as laminin-411, -511, collagen IV and nidogen. Laminin-411 has been identified as an important structural and signaling molecule in the vascular BM. To investigate the role of laminin-411 in inflammatory cell recruitment, we used several *in vivo* recruitment models and compared responses in WT and Lam4-/- mice.

Two different approaches were used to stimulate leukocyte extravasation in the mouse ear skin. In the first setting, croton oil was topically applied to the skin of the ear. Croton oil is known to trigger an unspecific inflammatory reaction that involves recruitment of different leukocyte subclasses. In a second approach, a DTH reaction was induced in the ear skin. Local challenge following sensitization with DNFB results in preferential recruitment of lymphocytes (Phanuphak *et al.*, 1974). In both models we found that the ratio between right and left ear weight was significantly smaller in the Lam4-/- mice compared to WT indicating a reduced inflammatory response (Figure 2A,B). As different types of leukocytes may use discrete mechanisms for extravasation, we applied additional recruitment models, the subcutaneous air pouch and thioglycollate-induced pleurisy to investigate monocyte and neutrophil recruitment, respectively. Monocyte recruitment to the air pouch stimulated by the chemoattractant MCP-1 was significantly attenuated in Lam4-/- mice, clearly indicating that laminin-411 is required for effective monocyte extravasation (Figure 2C). PMN recruitment as a response to thioglycollate-induced pleurisy was significantly reduced in mice lacking laminin-411 compared to WT mice (Figure 2D).

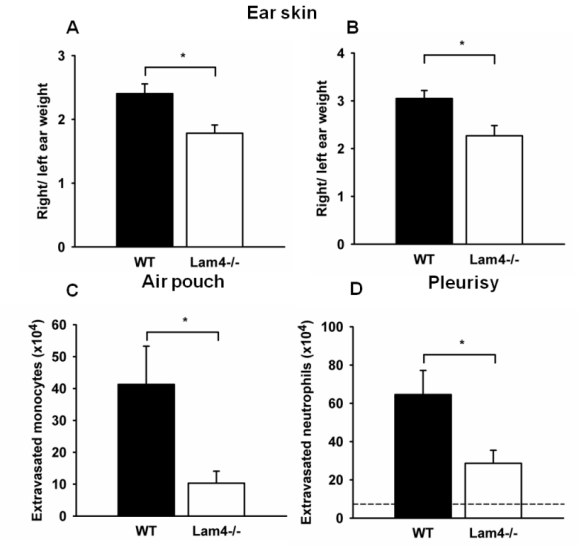
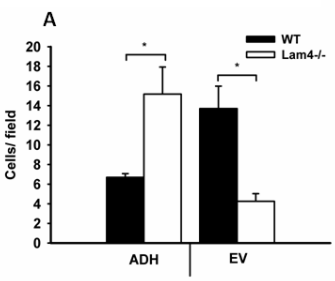
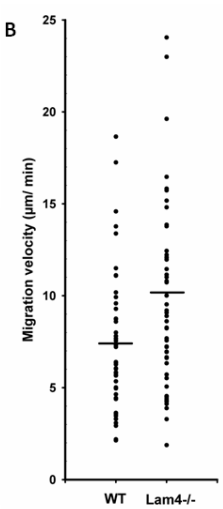


Figure 2. Leukocyte recruitment is impaired in Lam4-/- mice. A, B) Local skin inflammation in the ear was induced by topical application of croton oil (A, n=4) or challenge with DFNB in sensitized animals (B, n=6). The inflammatory response was assessed as tissue swelling and presented as the ratio between right (inflamed) and left (control) ear weight in WT (filled bars) and Lam4-/- (open bars) mice. **C)** Monocyte recruitment to a subcutaneous air pouch 24 hours after stimulation with 100 ng rmMCP-1 in WT (n=5) and Lam4-/- (n=5) mice. **D)** Pleurisy was induced by injection of 100 µl thioglycollate into the pleural cavity in WT (n=7) and Lam4-/- (n=7) mice, and neutrophil accumulation was assessed four hours later. Stimulation with sterile PBS was used as negative control (dashed line). * indicates significant difference at P<0.05. Values are mean ± SEM.

We established that deficiency in laminin-411 affects recruitment of all three major leukocyte subsets. Further experiments were undertaken to identify which step in the extravasation cascade that was impaired. For this purpose we used intravital microscopy to view the interactions between leukocytes and endothelial cells in venules of the cremaster muscle allowing analysis of leukocyte adhesion to the endothelium, transendothelial migration, and interstitial migration. Confirming our initial data, there was a decrease in recruitment of leukocytes to the extravascular space in Lam4-/- mice (Figure 3A). There was however no reduction in the number of adherent cells in the mice lacking the laminin α4 chain, rather vice versa, indicating that the decrease in leukocyte recruitment is not due to a defect in adhesion but more

Figure 3. Attenuated leukocyte recruitment in Lam4-/- mice is due to impaired migration through the vessel wall. A) Leukocyte extravasation in the cremaster muscle was induced by local stimulation with 100 nM PAF in WT (n=4, filled bars) and Lam4-/- (n=5, open bars) mice. Adherent (ADH) and extravasated (EV) cells per field were determined by IVM after a 60 min stimulation period. * indicates significant difference at P<0.05. Values are mean ± SEM. **B)** Migratory speed of emigrated cells was measured using intravital time-lapse video microscopy for 60 min in WT (n=3, 44 cells) and Lam4-/- (n=3, 50 cells) mice.





likely related to impaired migration across the vessel wall. Yet another cause for reduced leukocyte recruitment could be less efficient migration in the extravascular tissue (Lindbom & Werr, 2002). However, we did not find a reduction in migration velocity in the Lam4-/- mice compared to WT indicating that the locomotor response was intact (Figure 3B). Collectively, the findings obtained by IVM show that intravascular adhesion to the endothelial lining as well as migratory capacity in the extravascular tissue is not compromised in Lam4-/- mice, suggesting an impaired ability to penetrate the vessel wall in these mice as a principal cause for reduced leukocyte recruitment.

That loss of a structural component of the vascular basement membrane results in a less effective transmigration may seem surprising as one might expect that an incomplete barrier would rather facilitate cell passage. However, there are data to suggest that interaction with laminin-411 may positively influence leukocyte motility. Mononuclear leukocyte migration has previously been found to be more vigorous on laminin-411 than on laminin-511 (Pedraza *et al.*, 2000; Geberhiwot *et al.*, 2001; Wu *et al.*, 2009). We compared migration of neutrophils on these two laminin isoforms but there was no preferential migration of this leukocyte subset on laminin-411 relative to laminin-511. On the other hand, we found that neutrophil adhesion to laminin-511 under stationary conditions was enhanced compared to laminin-411.

The mechanism behind decreased recruitment in laminin α4 chain deficient mice remains unclear, but there are several possible explanations. Similar to what was reported for tumor cell migration (Vainionpaa *et al.*, 2007) it is conceivable that recognition of laminin-411 in the subendothelial basement membrane and signaling via laminin-binding receptors, such as integrin $\alpha_6\beta_1$, stimulates detachment of the rear of the leukocyte from the endothelium and thereby promotes forward migration through the vessel wall. This hypothesis is consistent with our finding of an increased number of adherent leukocytes to EC in Lam4-/- mice.

Laminin-511 has, contrary to the indicated role of laminin-411, been suggested to exert a restraining influence on leukocyte migration through the vessel wall. It was shown that leukocytes preferentially penetrate the basement membrane at sites low in expression of laminin-511 (Sixt *et al.*, 2001; Wang *et al.*, 2006; Voisin *et al.*, 2010). As mice lacking the laminin α4 chain reportedly exhibit a more uniform expression of laminin-511 along the vessel wall (Wu *et al.*, 2009), a shift in the distribution between these laminin isoforms could potentially explain the suppressed recruitment in Lam4-/- mice. Another possibility lies in the structure of the laminin α4 chain. Contrary to the other laminin α chains, the α4 chain is truncated which may reduce cross-linking with collagen IV (Hallmann *et al.*, 2005). This might facilitate migration through basement membranes high in laminin α4 chain as they are not as tightly intertwined. It was, however, not possible to detect any overt change in the distribution of Laminin-511 when comparing our histology specimens from WT and Lam4-/- mice. The postulated contrasting roles of laminin-411 and laminin-511 may seem surprising as the interaction between leukocytes and both laminin isoforms is mediated chiefly by the $\alpha_6\beta_1$ integrin (Geberhiwot *et al.*, 2001; Gu *et al.*, 2003). On the other hand, recent data show that migration across laminin-411 is inhibited with antibodies blocking the α_6 and β_1 integrins, whereas migration over laminin-511 is unaffected by this treatment (Wu *et al.*, 2009).

Neutrophils induce alterations in vascular permeability during acute inflammation (paper II and III)

It has been known for long that there is an increase in vascular permeability in conjunction with PMN recruitment in acute inflammation (Wedmore & Williams, 1981). The lipid mediator LTB_4 is a potent chemoattractant for PMN, and is known to stimulate enhanced vascular permeability through triggering PMN extravasation (Bjork *et al.*, 1982; Rosengren *et al.*, 1991). Neutrophil activation and release of granule protein HBP has previously been suggested to be a key mechanism behind PMN-induced changes in vascular permeability (Gautam *et al.*, 2001). However, the link between these two mediators is unknown and it was of interest to investigate a possible cross-talk between them.

Western blot analysis showed that stimulation of PMN with LTB_4 induced an immediate release of HBP. Degranulation of PMN as a result of LTB_4 stimulation is mediated by the BLT1 receptor as HBP release was completely inhibited in PMN treated with an antagonist to the BLT1 receptor. Neutrophil degranulation is known to involve different kinases such as p38 mitogen-activated protein-kinase and 1-phosphatidylinositol 3-kinase (PI3K) (Mocsai *et al.*, 2000; Gaudreault *et al.*, 2005; Nanamori *et al.*, 2007). We show that the BLT1 receptor signals via the Src family kinases and more specifically PI3K, which result in degranulation and the release of HBP.

The bioactivity of the PMN supernatant was tested on cultured ECs. Supernatant from LTB_4 stimulated PMN triggered a rise in EC $[Ca^{2+}]_i$. This response was attenuated in supernatant from PMN that were treated with a BLT1 receptor antagonist and supernatant where HBP was removed by immunoadsorption (Figure 4A). To further examine the relationship between LTB_4 challenge and PMN degranulation *in vivo*, pleurisy was induced in mice through injection of LTB_4 in the pleural cavity. The plasma extravasation induced by LTB_4 stimulation was abolished in mice that were depleted of PMN indicating that the response was PMN-dependent and that LTB_4 alone does not affect the endothelial cell permeability. Treatment of mice with a BLT1 receptor antagonist also attenuated this response. Intrapleural injection of the same supernatants as used for EC Ca^{2+} mobilization experiments showed that

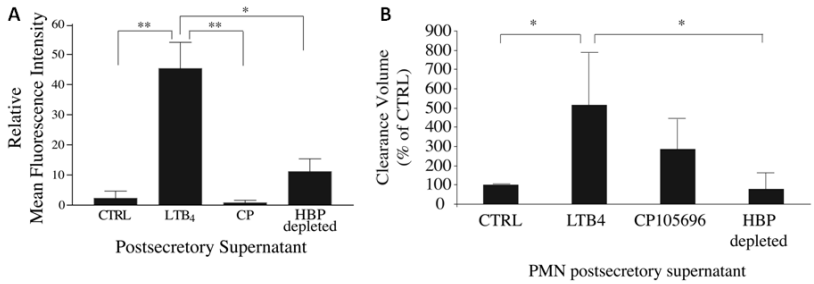


Figure 4. HBP released from LTB_4-treated PMN induce intracellular calcium mobilization *in vitro* and increased vascular permeability *in vivo*. A) Supernatant (50 µl) from untreated PMNs (CTRL; n=3), LTB_4-treated PMNs (LTB4; n=3), CP105696-pretreated PMNs (CP, CP105696; n=3), or supernatant depleted of HBP (HBP depleted; n=3) was added to microwells of endothelial cell monolayers, and intracellular Ca^{2+} mobilization was monitored. Data are expressed as relative MFI. **B)** The same supernatans (100 µl) were injected into the pleural space in PMN-depleted mice (n=4). After 4 h, exudate in the pleural cavity was collected for assessment of plasma extravasation (expressed as plasma clearance volume of intravenously injected FITC-dextran). Values are mean ± SEM. * indicates significant difference at $P<0.05$, and ** at $P<0.01$.

supernatant from LTB_4-treated PMN induces a significant increase in vascular permeability. Moreover immunoadsorption of HBP rendered the postsecretory supernatant inactive with respect to causing a change in vascular permeability (Figure 4B). These data agree with results obtained in EC $[Ca^{2+}]_i$ mobilization experiments and confirm that HBP released from LTB_4-stimulated PMN is not only biologically active but may also contribute to enhanced plasma extravasation during leukotriene-dependent inflammation.

To further clarify the mechanism how neutrophil granule proteins induce increases in vascular permeability it was of interest to investigate the involvement of the kallikrein-kinin system. Components of the kallikrein-kinin system, i.e. PK, FXII, and HK, assemble on the surface of EC and PMN where these proteins may be susceptible to attack from granule proteins released from PMN as they adhere to EC. Initial experiments aimed at elucidating whether PMN are capable of triggering activation of the kallikrein-kinin system on the EC surface. Endothelial cells were incubated with plasma, which resulted in binding of HK, and subsequently exposed to PMN that in some samples were activated with IL-8. Incubation with PMN alone or to a greater extent PMN stimulated with IL-8 triggered HK breakdown (Figure 5A). Kaolin, which results in a complete proteolysis served as a positive control. These data indicate that stimulated PMN can initiate activation of the kallikrein-kinin system leading to the proteolysis of high molecular weight kininogen.

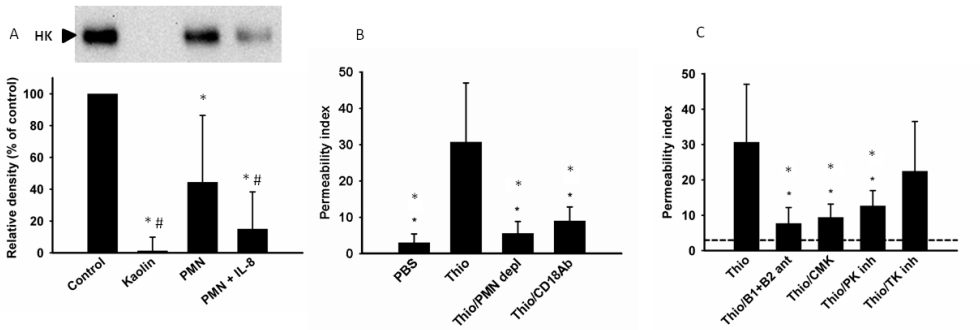


Figure 5. Neutrophil-induced plasma exudation is the result of degradation of high molecular weight kininogen. A) Upper panel; representative Western blot of HK. Lower panel; density quantification of Western blot as percentage of control (HUVEC treated with plasma). HUVEC were incubated with human plasma (lane 1, n=11) and treated with kaolin (lane 2, n=11), PMN (lane 3, n=7) or PMN stimulated with IL-8 (lane 4, n=11). Values are mean ± SD. * represents significant difference from control, # represents significant difference from PMN. **B, C)** Plasma exudation, represented as permeability index, in mouse pleurisy following treatment with (B) PBS (negative control, n=5), thioglycollate (Thio, n=7), anti-Gr1 antibody (PMN depl, n=5) and anti-CD18 antibody (CD18Ab, n=5). (C) Interference with the kallikrein-kinin system was performed using kinin B1 and B2 receptor antagonists (B1 + B2 ant, n=5), CMK (n=6), plasma kallikrein inhibitor (PK inh, n=8), or tissue kallikrein inhibitor (TK inh, n=8). Dashed line represents values for PBS treatment. Values are mean ± SD. * represents significant difference from Thio.

Additional investigations of the role of the kallikrein-kinin system in neutrophil evoked plasma exudation *in vivo*, used the mouse pleurisy and the hamster cheek pouch models. Thioglycollate-induced pleurisy resulted in profound plasma exudation, which was neutrophil-dependent (Figure 5B). Combined treatment with kinin B1 and B2 receptor antagonists largely prevented the PMN-induced plasma leakage. Treatment with two different PK inhibitors, H-D-Pro-Phe-Arg-chloromethylketone (CMK) or VA999026, to prevent kinin formation was almost as

effective as the kinin receptor blockade in preventing the PMN-evoked plasma exudation (Figure 5C). These findings were confirmed in the hamster cheek pouch model. The increase in vascular permeability caused by activated PMN was annulled by combined treatment with kinin B1 and B2 receptor antagonists. Additionally, in hamsters pre-treated with the specific PK inhibitor VA999026, the increase in venular permeability evoked by adherent PMN was essentially abolished. The cheek pouch venules were, however, still responsive to bradykinin (Figure 6A). Importantly, the number of adherent leukocytes in venules was not significantly reduced by treatment with either the PK inhibitors or the kinin receptor antagonists (Figure 6B) indicating that the suppression of plasma leakage in the *in vivo* models is not due to decreased leukocyte adhesion as a result of treatment with inhibitors.

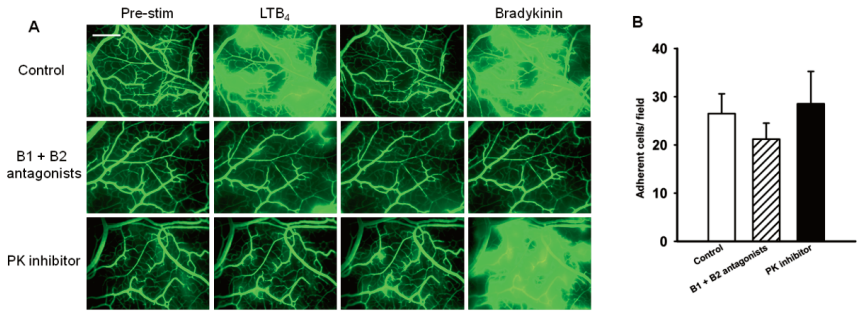


Figure 6. PMN-induced plasma leakage *in vivo* is attenuated following inhibition of plasma kallikrein. A) Representative *in vivo* micrographs showing plasma leakage in the hamster cheek pouch before (Pre-stim), 10 min after topical LTB_4 stimulation (LTB_4), following a 15 min wash period (Recovery), and after bradykinin stimulation (Bradykinin). Images were taken during control conditions (Control, n=3), following treatment with B1 and B2 receptor antagonists (n=3) or a PK inhibitor (n=3). Scale bar, 500 µm. **B)** Adherent cells following LTB_4 treatment. Values are mean ± SD.

It was previously reported that ligation of β_2 integrins triggers PMN degranulation and release of granule proteins, which stimulate cytoskeletal rearrangement and hyperpermeability in adjacent EC (Gautam *et al.*, 2000; Gautam *et al.*, 2001). Here we show that stimulation of PMN with the known chemoattractant LTB_4 results in a supernatant with the same ability to activate EC. This was mainly attributed to the presence of HBP. Further, we provide evidence for a pivotal role of the kallikrein-kinin system in the paracrine signaling pathway that links PMN recruitment with derangement of the endothelial barrier in inflammatory reactions. Originally, this cascade was shown to be initiated during contact of blood with negatively charged artificial surfaces (Colman & Schmaier, 1997). Although similar activation may occur in contact of blood with subendothelial collagens or bacterial membranes *in vivo*, a physiologic mechanism for triggering the kallikrein-kinin cascade has remained elusive. Our present findings suggest that PMN activation and adhesion to the endothelial lining constitutes a physiologic basis for intravascular kinin formation. However, the activation pathway remains unknown and there are several possible mechanisms for neutrophil-induced HK breakdown.

Heparan sulfate and related glycosaminoglycans have been suggested to anchor HK on endothelial cells (Renne *et al.*, 2000; Schmaier, 2008), and it appears that HK is protected from proteolytic processing while it is bound to the cell surface glycosaminoglycans (Renne *et al.*, 2005). This inhibitory shield can be overcome by competitive displacement of HK (Renne *et al.*, 2005). Possibly, PMN granule proteins, e.g. HBP, stimulate displacement of HK from the EC surface because of charge interactions. HBP carries a large number of positively charged amino acid residues concentrated to one side of the protein (Iversen *et al.*, 1997), creating a strong basic patch that favors binding to negatively charged proteoglycans. Therefore, the mechanism behind PMN granule protein activation could be an interference with HK binding to EC thus allowing for proteolytic cleavage into active kinin fragments. A charge-dependent interaction is consistent with the capacity of anionic compounds to inhibit increases in permeability caused by activated neutrophils (Rosengren *et al.*, 1989; Gautam *et al.*, 2000).

An alternative pathway for activation of the kallikrein-kinin system by PMN may be related to the proteolytic activity of PMN granule proteins, as has been suggested previously (Stuardo *et al.*, 2004). Specifically neutrophil elastase and proteinase 3, which are located in the primary granules (Borregaard *et al.*, 2007), have been shown to cause kininogen hydrolysis (Imamura *et al.*, 2002; Kahn *et al.*, 2009). However, of principal physiological relevance is the fact that HBP, in addition to being stored in the primary granules, is discharged from secretory vesicles in close proximity to the neutrophil plasma membrane (Tapper *et al.*, 2002). While elastase and related neutrophil-derived proteases are only slowly mobilized from the primary and secondary granules, storage of HBP in secretory vesicles allows for its instantaneous release upon cell activation and adhesion to the endothelial lining, which fits with the kinetics of neutrophil-evoked alterations in EC barrier function (Gautam *et al.*, 1998).

Previous data have shown that activated neutrophils trigger intracellular Ca^{2+} mobilization, remodeling of the actin cytoskeleton and increased paracellular permeability in cultured EC largely identical to the responses achieved by directly-acting agonists such as bradykinin (Lindbom, 2003), which is generated by activation of the kallikrein-kinin system (Schmaier, 2008). The similarity in the EC responses to activated PMN and direct agonist stimulation is explained by our findings as we show that these two, previously not linked, pathways converge at the endothelial cell surface through the generation of bradykinin.

Neutrophil recruitment and increased vascular permeability are related but dissociated events (paper II and III)

The close connection between neutrophil recruitment and plasma extravasation, which has been shown in several studies (Wedmore & Williams, 1981; Arfors *et al.*, 1987) makes it reasonable to hypothesize that plasma leaks from the blood stream as the neutrophil migrates across the endothelium. However, we show that neutrophil recruitment and plasma extravasation can be dissociated events. The mouse pleurisy model allows for simultaneous assessment of leukocyte accumulation and exudate formation and this model was used in Lam4-/- mice as well as WT mice that had been treated with inhibitors to the kallikrein-kinin system. When pleurisy is induced by thioglycollate, the edema formation is neutrophil-dependent. It was found that mice deficient in the laminin α4 chain exhibited a decrease in PMN recruitment but had a

normal exudate formation compared to WT mice (Figure 2D and 7A respectively). On the contrary, WT mice treated with inhibitors of the kallikrein-kinin system had attenuated plasma extravasation but there was no effect on neutrophil extravasation (Figure 5C and 7B respectively).

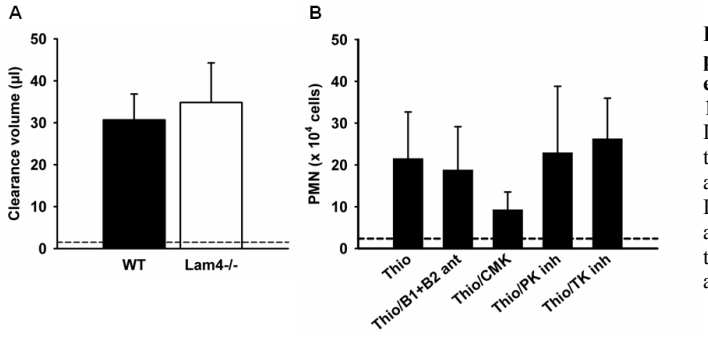


Figure 7. Neutrophil accumulation and plasma leakage are related, but dissociated events. Pleurisy was induced by injection of 100 µl thioglycollate into the pleural cavity. Dashed line represents values for PBS treatment. **A)** Plasma extravasation calculated as clearance volume in WT (n=7) and Lam4-/- (n=7) mice. **B)** Neutrophil accumulation in mice treated with inhibitors to the kallikrein-kinin system (n=5-8). Values are mean ± SEM (A) and SD (B).

Few studies have attempted to investigate a dissociation between neutrophil recruitment and vascular permeability using inhibitors or genetically modified mice. However, Zeng et al. reported that leukocyte adhesion and emigration not always cause a corresponding increase in vascular permeability and conclude that the mechanisms that regulate leukocyte recruitment must be different from those controlling endothelial barrier function (Zeng *et al.*, 2002). Further, inhibition of inducible NO synthase attenuates increased vascular permeability without an effect on PMN recruitment in a model of acute lung injury induced by intestinal ischemia/ reperfusion (Cavriani *et al.*, 2004). Reduced vascular permeability without altered adhesion or emigration of leukocytes was also shown in the LTB_4 stimulated hamster cheek pouch preparation after treatment with dextran sulphate, and the authors speculated that dextran sulphate might interfere with neutrophil granule proteins (Rosengren *et al.*, 1989).

Neutrophil granule proteins are released when the cell adheres to the endothelium and have been shown to increase vascular permeability (Gautam *et al.*, 2000). In accordance, the response seen in Lam4-/- mice suggests that adhesion to the endothelium rather than transmigration through the vessel wall is important for increased vascular permeability. Another possibility for the dissociation could be the adhesive interaction and ligation of EC adhesion molecules that causes EC activation, not paracrine signaling via granule proteins. However, this is disputed by the lack of effect on adhesion (c.f. Figure 6B) or recruitment in the animals treated with inhibitors of the kallikrein-kinin system despite attenuated permeability changes. Therefore neutrophil granule proteins released primarily from secretory vesicles appears as the most probable mechanism for altered endothelial barrier function. The kallikrein-kinin system could serve an essential link between these two events.

Neutrophils play a role in edema formation and tissue loss following traumatic brain injury (paper IV)

Leukocyte-induced adjustment of endothelial barrier capacity represents an important regulatory mechanism in immune surveillance allowing blood components to leave the vasculature and enter into extravascular tissue. However, derangement of the endothelial barrier due to neutrophil activation may be detrimental and even fatal in diverse inflammatory conditions such as infectious and allergic disease, ischemia-reperfusion, sepsis, and traumatic brain injury. TBI is associated with inflammatory responses leading to BBB dysfunction and edema formation, activation of resident and recruitment of bloodborne leukocytes and impaired function (Lenzlinger *et al.*, 2001). Severe TBI continues to be a leading cause of death and morbidity. Systemic and intracranial physiologic management, such as fluid resuscitation and intracranial pressure monitoring contribute to the greatest impact on survival. However, to minimize the secondary injury, pharmacological intervention will be critical in future treatments and it is therefore important to understand the mechanisms behind secondary injury following TBI. Hence, it was therefore of interest to investigate the role of PMN in TBI, specifically in the edema formation.

Neutrophil recruitment and edema formation are key events following traumatic brain injury. However, the connection between the two phenomena is not clear. Increased brain water content is an important clinical outcome of TBI as increased intracranial pressure may result in impaired blood supply and oxygen delivery to the parenchymal tissue (Unterberg *et al.*, 2004). We used two methods to determine edema formation following TBI caused by CCI; assessment of brain water content and Evan's blue extravasation. Brain water content has been shown to increase following TBI and remain increased for up to 7 days (Hellal *et al.*, 2004). Here, we show that this increase 48 hours after injury is attenuated in mice that are depleted of neutrophils indicating a role for PMN in the edema formation (Figure 8A). In order to assess disruption of the BBB, we used Evan's blue as a marker for macromolecule extravasation four and 24 hours after injury. CCI resulted in an increased EB

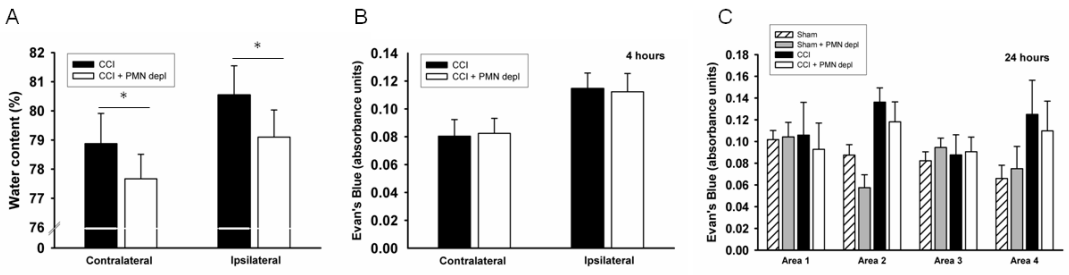


Figure 8. Brain water content, but not macromolecule extravasation, following TBI is affected by neutrophil depletion. A) Brain water content in the contralateral and ipsilateral areas 48 hours after CCI in intact (black bars) and PMN-depleted (open bars) mice. **B)** Evan's blue content in the contralateral and ipsilateral areas at 4 hours after CCI in intact (black bars) and PMN-depleted (open bars) mice. **C)** Mice were sham or CCI operated and neutrophils were left intact (sham: hatched bars, CCI: black bars) or depleted (sham: grey bars, CCI: open bars), and Evan's blue content was measured 24 hours later in the four different areas of the brain. Area 2 is the injured site. * indicates significant difference at $p < 0.05$. Values are mean ± SD.

extravasation in the injured area compared to the contralateral side or sham-operated animals at four and 24 hours after injury (Figure 8B and 8C). There was a tendency (p=0.081) for an attenuation of EB content on the injured side (area 2) in the PMN-depleted animals 24 hours but not four hours after CCI. Taken together, these data indicate that neutrophils contribute to brain edema but not necessarily through disruption of the BBB.

Our findings here could clarify the inconsistencies in previous research regarding the role of PMN in brain edema formation, as the time point for measurement seems to affect the results. For example, a previous study which could not confirm a role of neutrophils in TBI measured edema formation at four hours after trauma (Whalen *et al.*, 1999). We show similar results. Possibly, permeability changes due to the direct mechanical insult caused by CCI mask effects of neutrophil activation at early time points. Hemoglobin values have been shown to be the highest four hours after injury (Hellal *et al.*, 2004) indicating bleeding and large openings in the BBB. Another reason for differing results may be that EB extravasation is a measure of macromolecular efflux across BBB, whereas fluid can accumulate in brain tissue for other reasons without an increase in vascular permeability. The predominant form edema responsible for brain swelling and increased intracranial pressure following TBI has been shown to be cellular and not vasogenic (Beaumont *et al.*, 2000; Marmarou *et al.*, 2006), which may in part explain our results. The role for PMN could also be time dependent with PMN being more important at later time points. The exact mechanism of how neutrophils may cause edema formation following traumatic brain injury is not entirely understood. Possible mechanisms include decreased BBB function, increased breakdown of cells leading to cytotoxic edema, and the production and release of oxygen radicals and proteases.

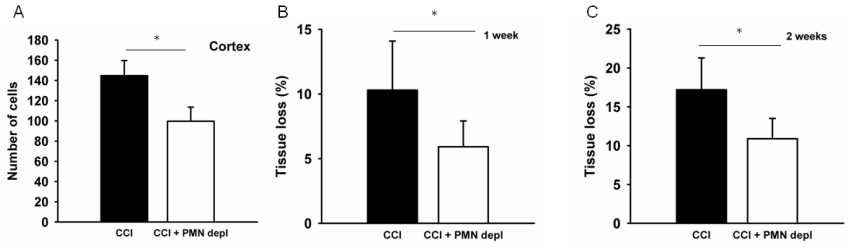


Figure 9. Neutrophil depletion attenuates microglial activation and tissue loss following TBI. A) Quantification of Mac-2 positive cells. **B, C)** Tissue loss one (A) and two (B) weeks after TBI in mice with intact PMN (black bars) and mice that were rendered neutropenic (open bars). ***** indicates significant difference at $p < 0.05$. Values are mean ± SD.

We show that microglial activation is less prominent in mice that are rendered neutropenic. In the cortex there was a large number of activated microglia after TBI which was significantly reduced in the PMN-depleted group (Figure 9A). In neutrophil-depleted mice there was a significant reduction of tissue loss compared to intact mice both at 7 and 14 days after injury (Figure 9B,C). Thus, there is a beneficial effect of PMN depletion in the later phase of the injury development, possibly due to less edema or a reduced number of inflammatory cells. Decreased microglia activation and tissue injury as a result of neutrophil depletion might be due to an

attenuation of the secondary injury caused by PMN. Another possibility could be that recruited PMN release inflammatory mediators and granule proteins which activate microglia and cause damage to the parenchymal cells. It has previously been shown that blocking ICAM-1, thus reducing PMN recruitment, results in improved neurological scores following brain injury (Knoblach & Faden, 2002). PMN may aggravate the injury by several mechanisms; especially their ability to secrete matrix metalloproteinases (MMPs), reactive oxygen species and cytokines have been implicated in this respect (Nguyen *et al.*, 2007). Inhibiting any of these factors were shown to be neuroprotective in *in vivo* models of TBI (Lewen *et al.*, 2000; Wang *et al.*, 2000; Bermpohl *et al.*, 2007), making the assumption that PMN activation and infiltration is involved in the secondary injury after TBI highly plausible.

CONCLUDING REMARKS

The following conclusions can be drawn based on the experimental evidence presented in this thesis:

1. Lack of laminin-411 in the perivascular basement membrane leads to impaired leukocyte migration through the vessel wall indicating that this laminin isoform is required for efficient recruitment of leukocytes in inflammation.

2. Leukotriene B_4 evokes increased vascular permeability and plasma exudation through inducing neutrophil degranulation and release of heparin binding protein.

3. Neutrophil-induced increase in vascular permeability involves activation of the kallikrein-kinin system and can be inhibited without affecting cell recruitment.

4. Neutrophils contribute to edema formation and tissue damage following traumatic brain injury.

These findings imply a significant role for neutrophils in inflammatory edema formation, which constitutes a significant clinical problem in disease conditions such as sepsis, brain trauma and different forms of pulmonary inflammation. Although it might be tempting to suggest that inhibition of PMN function in acute inflammatory reactions would have beneficial effects overall, this may not be the case. Neutrophils are known to cause tissue damage through the release of ROS and proteases, but are also absolutely essential in normal host defence. They are equipped with an array of antimicrobial proteins and have a phagocytic machinery of fundamental importance in clearing microbial infections, and they interact with other leukocytes to regulate and adjust the immune response. The ability to separately control neutrophil recruitment on the one hand and alteration of vascular permeability caused by the activated neutrophils on the other, with for example inhibitors to granule proteins, or the kallikrein-kinin system, allows for selective inhibition of edema formation while maintaining mobilization of these protective immune cells.

Both neutrophil-derived HBP and the kallikrein-kinin system have been implicated in the acute inflammatory reaction associated with sepsis. As suggested by the findings in this thesis, there is a close interaction between these mediators. Using this knowledge, future experiments could possibly identify novel targets to decrease the severity of sepsis. Further, the kallikrein-kinin system has been implicated in the mechanism behind edema formation following TBI, and especially inhibition of the B2 receptor has been shown to reduce edema formation. Therefore, in light of the findings on involvement of the kallikrein-kinin system in PMN-dependent alterations in vascular permeability, it is of interest to more thoroughly investigate the role of the neutrophil-kinin axis in TBI.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Inflammation är viktigt för läkning efter infektion eller skada. Vid inflammation rekryteras vita blodkroppar från blodet till den skadade vävnaden. De fastnar först på de celler som sitter på insidan av blodkärl, endotelceller, i närheten av det infekterade eller skadade området och vandrar sedan igenom kärlväggen. Under denna rekryteringsprocess kommer de vita blodkropparna skapa ett läckage av vätska och proteiner ut från blodet vilket leder till svullnad. En svullnad kan hindra det dagliga livet om den uppstår vid till exempel en knä- eller fotled, men kan vara livshotande vid. Experimenten i den här avhandlingen var utformade för att försöka förstå hur de vita blodkropparna tar sig från blodet ut till ett sår eller en skada, och varför en svullnad uppstår samtidigt.

För att likna inflammationsprocessen användes vita blodkroppar och endotelceller från människa, samt modeller i möss. Resultat från experimenten visade att de vita blodkropparna behöver känna igen ett protein kallat Laminin-411 för att kunna ta sig igenom kärlväggen. Detta protein sitter under endotelcellerna och hjälper blodkärlet att få rätt form. Möss utan Laminin-411 visar en minskad rekrytering av vita blodkroppar, d.v.s. färre celler tar sig från blodet till en skada eller infektion. LTB_4 är en substans som släpps ut från celler vid en infektion och drar vita blodkroppar till sig. Mänskliga vita blodkroppar som inkuberades med LTB_4 släppte ut ett ämne kallat HBP. HBP kan i sin tur göra så att det läcker ut vätska och protein från blodet vilket leder till svullnad. Vidare visades att svullnaden som uppstår när de vita blodkropparna tar sig från blodet är beroende av bradykinin som är ett ämne som frisätts vid inflammation. I möss som behandlades mot bradykinin kunde de vita blodkropparna ta sig ut från blodet utan att det bildades en svullnad. Eftersom de vita blodkropparna behövs för läkning så kan det vara önskvärt att stoppa svullnaden utan att minska antalet vita blodkroppar som lämnar blodet. Slutligen testades rollen för de vita blodkropparna i möss som utsattes för traumatisk hjärnskada. Möss som behandlades så att vita blodkroppar saknades i blodet fick mindre svullnad i hjärnan och även en minskad skada.

För att kunna motverka svullnad vid inflammation är det viktigt att förstå hur den uppkommer. Resultaten i den här avhandlingen kan förhoppningsvis bidra till att utveckla läkemedel som kan användas vid inflammation för att förbättra prognosen för patienter med sjukdomstillstånd som ger svullnad i hjärnan eller lungorna.

LIST OF ABBREVIATIONS

AJ	Adherens junction
BBB	Blood-brain barrier
BM	Basement membrane
C5a	Complement factor 5a
$[Ca^{2+}]_i$	Intracellular Calcium concentration
CCI	Controlled cortical impact
CMK	H-D-Pro-Phe-Arg-chloromethylketone
CNS	Central nervous system
DTH	Delayed time hypersensitivity
DNFB	1-Fluoro-2,4-dinitrobenzene
EC	Endothelial cell
ESAM	Endothelial cell selective adhesion molecule
FITC	Fluorescein isothiocyanate
fMLP	Formyl-Methionyl-Leucyl-Phenylalanine
FPRL1	Formyl Peptide Receptor Like-1
FPR1	Formyl Peptide Receptor 1
FXII	Factor XII
GPCR	G protein coupled receptor
HBP	Heparin binding protein
HK	High molecular weight kininogen
HUVEC	Human umbilical vein endothelial cells
ICAM-1	Intercellular adhesion molecule-1
IL	Interleukin
IVM	Intravital microscopy
JAM	Junctional adhesion molecule
LK	Low molecular weight kininogen
LT	Leukotriene
LTB_4	Leukotriene B4
MCP-1	Monocyte chemotactic protein-1
MMP	Matrix metalloprotease
MPO	Myeloperoxidase
MLC	Myosin light chain
MLCK	Myosin light chain kinase
MLCP	Myosin light chain phosphatase
NO	Nitric oxide
p120	p120 catenin
PAF	Platelet activating factor
PAR	Proteinase activated receptors
PECAM-1	Platelet/ endothelial cell adhesion molecule-1
PGE_2	Prostaglandin E2
PI3K	1-phosphatidylinositol 3-kinase
PK	Plasma kallikrein
PKC	Protein kinase C
PMN	Polymorphonuclear leukocyte
ROCK	RhoA kinase
ROS	Reactive oxygen species

SNARE	Soluble NSF attachment receptor
TBI	Traumatic brain injury
TJ	Tight junction
TNF	Tumor necrosis factor
VCAM-1	Vascular cell adhesion molecule-1
VE-cadherin	Vascular endothelial-cadherin
VE-PTP	Vascular endothelial protein tyrosine phosphatase
VEGF	Vascular endothelial growth factor
ZO	Zonula occludens

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